THE RÔLE OF CARBOHYDRATES IN BIOLOGICAL OXIDATIONS AND REDUCTIONS. EXPERIMENTS WITH PNEUMOCOCCUS

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INTRODUCTION

When repeatedly washed in saline solution, living cells lose in a large measure their ability to oxidize and to reduce. This fact was established by studies of oxygen consumption and methylene blue reduction with muscle tissues and yeasts. It led to the concept that the oxidation-reduction properties of a tissue or a cell are really due to the oxidation-reduction properties of certain metabolites after they have become "activated" by the tissue or cell under consideration.

The analysis of phenomena of this nature in bacterial systems was apparently begun by Harden and Zilva (1) in 1915. They found that washed cells of *B. coli* which reduce methylene blue only very slowly, reduce the same dye very rapidly in the presence of various substances. This work was much extended by Quastel and his associates (2) who referred to the washed cells as "resting bacteria." "Resting bacteria are simply bacteria in a state of non-proliferation and may be investigated in a manner similar to enzyme or catalytic systems." Working with *B. coli*, *B. pyocyaneus* and *B. alkaligenes*, they found that these organisms can activate the reduction of methylene blue by certain sugars, amino acids, and fatty acids (especially formic, lactic and succinic). *B. coli* was found to have an even wider range of activating action than muscle, 56 substances out of 103 studied being "activated" in this manner. Quastel (3) suggested that the process of "activation" consists in a polarization of the substrate molecules at particular regions or centers on the surface structures (interfaces) of the cell

In 1914, Cole (4) investigated the mechanism of the reaction whereby Pneumococci transform hemoglobin into methemoglobin. The reaction does not occur when hemoglobin is added to an emulsion of washed cells in salt solution. However, if minute traces of dextrose (or a number of other organic substances) be added to such a mixture, the reaction quickly occurs; it is always conditioned by the presence of oxygen. Avery and Neill (5) have described a large number of other oxidation-reduction processes exhibited by Pneumococcus cultures; amongst

which we shall consider in our studies the reduction of methylene blue and the formation of peroxide. The fact that these processes have the same optimum and limiting condition (temperature, pH, etc.,) suggests that they are brought about by one and the same system. They are exhibited not only by the whole intact cells, but also by sterile extracts of these cells prepared under proper conditions. They can also proceed under conditions of pH and temperature which do not permit active growth. As in the case of muscle, yeast and *B. coli*, the washed cells of Pneumococcus are unable to reduce, to consume oxygen, or to form peroxide. However, the oxidative-reductive properties can be restored by the addition to the washed cells of the cell washings, and of aqueous or alcoholic extracts of muscle, yeast and vegetable tissues. The oxidation-reduction system of the Pneumococcus cell therefore appears to consist of at least two components: 1) the heat stable component, just referred to, which can be readily washed out of the cell and is not necessarily of Pneumococcus origin. 2) a labile cellular component, not removed by washing, inactivated by 10 minutes heating at 65°C.

In the experiments to be presented here, we have attempted to analyze further the mechanism of bacterial oxidations and reductions and to establish new reactions of physiological significance. This report is limited to the influence of carbohydrates, especially glucose, as these substances appear to be of primary importance in the phenomena of growth. Although most of the work has been done with Pneumococcus cells, it is hoped that the facts established will prove to be of a more general significance in an understanding of cellular physiology.

EXPERIMENTAL

Experimental Methods

1. Bacteriological.—The experiments have been carried out with 3 strains of Pneumococcus: Type I (1/219/4), Type III (A/66/73) and an R cell derived from Type II (D/39/R). The results obtained apply to all 3 strains; for the sake of simplicity, all protocols refer to strain D/39/R.

Unless otherwise stated, very young cultures (6 hours old) were used in the tests; these cultures were obtained by seeding 2 cc. of a young culture into 150 cc. of meat infusion broth containing 1 per cent Witte's peptone and 0.03 per cent dextrose. The cells were separated from the medium and washed in physiological salt solution by centrifugalization (30 minutes—about 3000 rotations per minute).

By "sugar free meat infusion," we understand beef infusion prepared according to the standard method and from which the sugars have been removed by growing Pneumococcus in the infusion and filtering out the cells.

2. Chemical.—All the tests were performed in phosphate buffer solutions (0.03 molar) at pH 8.0.

The dye solutions were prepared as described in an earlier paper (6).

The following substances were used as source of oxidized thiol groups; a) glutathione (in the form of meat infusion); b) saturated cystine solution at pH 8.0; c) a product obtained by the auto-oxidation of thioglycollic acid (Eastman) in the presence of air, with sodium nitroprusside as a catalyst (in dilute ammonia solution); we shall refer to the last product as "oxidized thioglycollic acid" without attaching to this expression any real meaning concerning the exact nature of the compound.

The reduction experiments were carried out in Noguchi tubes under a vaseline seal of 2 inches thickness, at room temperature (about 20°C.). The nitroprusside test was used to detect the presence of reduced thiol groups. The formation of peroxide was tested by transferring 10 cc. of the systems under consideration to 150 cc. Erlenmeyer flasks which were incubated at room temperature; the titanium sulphate test was used for detecting the peroxide present.

TABLE I

Reduction of Methylene Blue by Pneumococcus Cultures at Different Periods of Growth

No. of hours after inoculation at which samples were taken	Time required for the reduction of 0.2 cc. of 0.0025 M methylene blue by 5 cc. culture	
	Plain broth culture	Glucose broth culture
1 hour	6 hours	6 hours
3 hours	4 "	3 "
4 " 30 minutes	2 " 20 minutes	1 " 5 minutes
6 "	2 " 10 "	0 " 30 "
7 " 30 minutes	4 "	0 " 25 "
12 "	8 "	0 " 30 "
24 "	18 "	2 " 10 "

The Reduction of Methylene Blue by Pneumococcus Cultures

When one attempts to determine the "reducing power" of a Pneumococcus culture, it is found that even under the same conditions of temperature and reaction, the rate of reduction of methylene blue varies a great deal, according to the composition of the medium and the age of the culture.

Experiment 1.—Two flasks, each containing 150 cc. of plain broth, were seeded with 2 cc. of a young culture of Pneumococcus. One of the flasks received in addition 0.1 per cent glucose. The two flasks were incubated at 37°C. and samples were removed after 1, 3, 4½, 6, 7½, 12 and 24 hours incubation. The reaction of these

samples was adjusted to pH 8.0 with NaOH and a reduction test was set up with 0.2 cc. of 0.025 M methylene blue. The times required for reduction are given in Table I.

Although no bacterial counts were made, the turbidity of the cultures indicated that the maximum growth was obtained after 6–8 hours incubation. The fact that during the early periods of incubation, the dye was more rapidly reduced in the glucose broth culture than in the plain broth culture may be accounted for by the more rapid multiplication of the organisms in the former medium. But the rapid decrease of the "reduction power" of the plain broth culture as compared with its constancy in the glucose broth culture cannot be attributed to the difference in the number of cells, for it is known that the number of R cells of Pneumococcus remain constant for at least 24 hours in plain broth cultures, while the same organisms begin to die off more rapidly in glucose broth. It appeared, therefore, that the constituents of the medium played an important rôle in the reducing power of the culture. This is shown by Experiment 2.

Experiment 2.—Flasks containing 150 cc. of plain broth were seeded with 1.25 cc. of a young D/39/R culture. 10 cc. samples were taken at different times after inoculation, and adjusted to pH 8.0. In each case, 2 samples received 0.1 cc. of a 1 per cent glucose solution and 2 were kept as control, one of the duplicate tubes in each series was placed in a bath of boiling water for 2 minutes. 2 cc. of 0.0025 M methylene blue was then added to each one of the tubes. The time required for reduction of the dye is given in Table II.

A number of conclusions can be drawn from this experiment: a) the addition of small amounts of glucose to Pneumococcus cultures brings about a more rapid reduction of the methylene blue; b) the decrease in the reduction power of a plain broth culture after the sixth hour of growth is probably due to the disappearance of the small amount of sugar originally present in the broth; c) the ability of the glucose to reduce methylene blue is probably due to some cellular factor, since it reaches its maximum at the time when the growth is maximum; d) this factor slowly loses its power as the culture ages.

It is known that oxidized methylene blue is bacteriostatic for Pneumococcus (7); this and the fact that the reduction of the dye occurs so rapidly (12 minutes) is an indication that the phenomenon of reduction

is not associated with a proliferation of the cells. The experiments with washed cells serve to demonstrate this point.

TABLE II

The Effect of Heating, Age of the Culture, and Addition of 0.1 cc. of 1 Per Cent Glucose on the Velocity of Reduction of 0.2 cc. of 0.0025 M Methylene Blue by Pneumococcus Cultures (10 cc.)

No. of hours after inoculation at which sample was taken	No. of cells per cc. of culture	Time required for reduction by:				
		Culture	Heated culture	Culture + dextrose	Heated culture + dextrose	0.1 cc. of 1 per cent dextrose
1 hour	10^7	12 hrs.	—*	12 hrs.	—	—
2 hours	10^8	9 hrs.	—	9 hrs.	—	
3 " 30 minutes	10^9	2 hrs. 15 min.	—	2 hrs. 15 min.	—	
5 "	10^9	55 min.	—	45 min.	—	
6 " 30 minutes	10^9	12 min.	—	10 min.	—	
8 "	10^{10}†	44 min.	—	13 min.	—	
10 "	10^{10}†	2 hrs. 30 min.	—	15 min.	—	
12 " 30 minutes	10^{10}†	6 hrs.	—	17 min.	—	
23 "	10^9	16 hrs.	—	27 min.	—	
26 "	10^9	—	—	45 min.	—	
30 "	10^8	—	—	48 min.	—	
47 "	10^6	—	—	6 hrs.	—	

* The sign — indicates that the dye was not reduced after 24 hours. The heated cultures, however, showed some reduction at that time. This reduction was of the same order as that exhibited by sterile media (6) and need not interest us here.

† The apparent increase in numbers observed after the 8th hour of incubation is probably due to the breaking up of the Pneumococcus chains

The Reduction of Methylene Blue by Washed Cells of Pneumococcus; Its Activation by Meat Infusion

Experiment 3.—A young culture of D/39/R in plain broth was centrifuglized, and the cells washed 7 times in saline. A sample corresponding to 15 cc. of culture was taken following each washing, and the ability of the washed cells to reduce methylene blue was tested in the absence and in the presence of glucose.

This experiment demonstrates that cells washed free of metabolites are unable to reduce methylene blue; however, they do reduce it rapidly

in the presence of glucose; the partial reduction obtained with the unwashed cells without further addition of sugar is to be traced to the metabolites associated with the cells and which are removed by repeated washings. It is also evident that, as the washings are repeated, the cells progressively lose their ability to reduce in the presence of

TABLE III

The Reduction of Methylene Blue (0.1 cc. of 0.005 M) by Washed Cells of Pneumococcus

No. of washings	Time required for complete reduction	
	No glucose	0.2 cc. of 0.005 M glucose
0†	—*	2 minutes
1†	—	5 "
2	—	9 "
3	—	16 "
4	—	29 "
5	—	40 "
6	—	—
7	—	—

* The sign — indicates that the dye was not reduced in 24 hours.

† The first and second samples of cells showed a partial reduction of the dye in 24 hours, even without glucose.

TABLE IV

Influence of Sugar-free Meat Infusion on the Velocity of Reduction of Methylene Blue by Washed Cells of Pneumococcus in the Presence of Glucose

Washed cells	Methylene blue 0.002 M	Glucose 0.002 M	Meat infusion	Time required for complete reduction
cc.	cc.	cc.	cc.	
10	1	1		90 minutes
10	1	1	0.5	40 "
10	1	0	0.5	Reduction only partial after 24 hours

glucose. Now, it had been observed in the course of previous experiments that the presence of sugar-free meat infusion increases the velocity of reduction of methylene blue by the Pneumococcus-glucose system, although a system containing washed cells and sugar-free meat infusion is unable by itself to bring about any *rapid* reduction. An example of such an action is given in Experiment 4.

Experiment 4.—A young plain broth culture of Pneumococcus was centrifugalized and the cells washed twice in saline. The washed cells were suspended in buffer pH 8.0 and their reducing power tested with 1 cc. of 0.002 M methylene blue and 1 cc. of 0.002 M glucose—with and without the addition of 0.5 cc. sugar-free meat infusion. A tube containing 10 cc. of cells + 0.5 cc. meat infusion + 1 cc. of 0.002 M methylene blue, but no glucose, was used as control (Table IV).

Since sugar-free meat infusion is found to increase the velocity of reduction of methylene blue by a system containing Pneumococcus + glucose, it appeared possible that the action of the cell on the glucose might depend on two factors, one which cannot be removed by washing and another present in meat infusion. Experiment 5 bears on this point.

TABLE V

Influence of Washing the Cells and of the Addition of Meat Infusion on the Reduction of Methylene Blue by the Pneumococcus-Glucose System

No. of washings	No meat infusion	0.5 cc. of meat infusion
1	+*	+
2	+	+
3	+	+
4	−*	+
5	−	+
6	−	+
7	−	−

* The signs + and − indicate that the methylene blue was, or was not completely reduced after 24 hours incubation.

Experiment 5.—Young cells of D/39/R were repeatedly washed in saline. At each one of the washings, experiments with amounts of cells corresponding to 10 cc. of culture were made to determine the reduction of 1 cc. of 0.001 M methylene blue by 1 cc. of 0.001 M glucose in the presence and in the absence of meat infusion (Table V).

The results of Experiment 5 confirm the view that in the mechanism responsible for the reduction of methylene blue in the presence of glucose there are at least two constituents involved; one present in meat infusion, readily washed out of the cells, and heat stable; the other persisting in the cell despite repeated washing but eventually lost as result of the process. It is not known as yet whether this sec-

ond factor is simply washed out, or destroyed following some injury caused to the cell in the process of washing. It has been found to be heat-labile and completely inactivated by 10 minutes exposure at 55°C.

Meat infusion is well known to contain reducing substances, and it is important to know to what extent methylene blue can be reduced by sugar-free meat infusion in the presence of Pneumococcus cells.

Experiment 6.—Young cells of D/39/R were washed four times in saline, and 5 cc. amounts were used to determine the comparative reduction of methylene blue in the presence of glucose, sugar-free meat infusion, and glucose + meat infusion. The details of the experiment are given in Table VI.

TABLE VI

The Activation of the Pneumococcus-Glucose System by Sugar-free Meat Infusion

Washed cells	Glucose 0.001 M	Meat infusion	Methylene blue (cc. of 0.001 M solutions)									
			0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
cc.	*cc.*	*cc.*										
5	0	0	±	−	−	−	−	−	−	−	−	−
0	1	0	−	−	−	−	−	−	−	−	−	−
0	0	5	−	−	−	−	−	−	−	−	−	−
5	1	0	+*	±*	−	−	−	−	−	−	−	−
5	0	5	+	+	±	−	−	−	−	−	−	−
5	1	5	+	+	+	+	+	+	+	+	+	+

* In this table the signs + and − indicate that the dye had or had not been completely reduced in 3 hours. ± indicates that the reduction was complete only after 24 hours.

The results of Experiment 6 demonstrate that the rapid reduction of methylene blue by a system containing washed cells + meat infusion + glucose cannot be accounted for by the reducing power only of any one of the three components of this system or even by any combination of two of these components. A rapid reduction of the dye requires the presence of all three of them.

The Existence of a Definite Ratio Between the Amounts of Glucose Used and of Methylene Blue Reduced

In the course of the reduction which has just been described, the methylene blue is reduced and the glucose oxidized. An attempt has

been made to determine the relative amounts of the two substances involved in the reaction. However, great difficulties have been encountered in obtaining a constant ratio, although in all instances the reduction of 1 mol of methylene blue required from 0.7 to 1 mol of glucose. The following experiment is a typical example of the results obtained in such determinations.

Experiment 7.—Young cells of D/39/R were washed once in saline. This was found sufficient to rid the cell of practically all the metabolites they contained.

TABLE VII

The Quantitative Relation Between the Amounts of Glucose Used and of Methylene Reduced

Amount of cells	Methylene blue (0.001 M solutions)	Time in minutes required for complete reduction of the dye in the presence of the following amounts of 0.001 M glucose solution										
		0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
cc.	*cc.*											
15.0	1	—*	—	—	—	—	—	—	—	360	45	60
10.0	1	—	—	—	—	—	—	—	240	—	330	120
5.0	1	—	—	—	—	—	—	—	—	—	—	120
2.5	1	—	—	—	—	—	—	—	—	—	—	145
15.0	0.1	—	60	x*	x	x	x	x	x	x	x	x
10.0	0.1	—	100	x	x	x	x	x	x	x	x	x
5.0	0.1	—	80	x	x	x	x	x	x	x	x	x
2.5	0.1	—	120	x	x	x	x	x	x	x	x	x

* The sign — indicates that the dye was not yet completely reduced after 12 hours incubation; x indicates that the test was not made.

Cells washed only once do not need meat infusion to be able to activate glucose. Varying amounts of these cells were used with varying concentration of glucose and of methylene blue for a reduction test. The details and results of the experiment are given in Table VII.

The results of this experiment demonstrate once again the reduction of methylene blue by Pneumococcus in the presence of glucose, and the lack of reduction in the absence of the sugar. It will be seen that, whereas 2.5 cc. of cells could reduce 1 cc. of the methylene blue solution in the presence of glucose, 15 cc. of the washed cells alone could not even reduce 0.1 cc. of the same dye solution. It seems true also that the ratio of glucose to methylene blue becomes narrower as larger

amounts of cells are used for the test; and this, as we have just seen, cannot be accounted for by the metabolites present in the cells. The change in the glucose-methylene blue ratio might conceivably be due to side-reactions which become manifest only when larger amounts of cells are used. In experiments not reported here, it has been established that the time required for the reduction of 1 cc. of 0.001 M methylene blue is not dependent upon the amount of glucose used, when this amount is larger than 1 cc. of 0.001 M solution. The fact that the reduction with 0.7, 0.8 and 0.9 cc. of the glucose solution takes

TABLE VIII

Time Required for the Reduction of rH Indicators by the Pneumococcus-Glucose System

Dye			Amount of cells			
Name	Concentration 0.01 M	rH	20 cc.	10 cc.	5 cc.	2.5 cc.
	cc.					
2 chloro indophenol..	0.1	21.8	Immediate reduction		6 minutes	30 minutes
1 naphthol-2 sulfonate indophenol...	0.1	18.1	"	"	10 "	30 "
Methylene blue.....	0.05	14.4	5 minutes	10 minutes	15 "	1 hour
Indigo tetrasulfonate.	0.1	12.5	16 hrs.	20 hrs.	2 days	2 days
Indigo trisulfonate...	0.1	11.3	2 days	8 days	Only partly reduced in 2 weeks	
Indigo disulfonate...	0.1	9.9	2 "	8 "	" "	" "

longer than with 1 cc. is another evidence that we are dealing with at least two different reactions.

In conclusion, it appears that, under the proper conditions, the reduction of one molecule of methylene blue requires one molecule of glucose.

The Reduction of the Indicators of Oxidation-reduction Potentials by the Pneumococcus-glucose System

The work of the past few years has indicated that many biological systems develop reducing potentials much lower than that of reduced

methylene blue. Experiment 8 demonstrates that when glucose is placed in the presence of Pneumococcus cells, dyes with a very low rH are also reduced.

Experiment 8.—A young culture of D/39/R was centrifugalized and the cell suspended in buffer at pH 7.8. Amounts of the suspension corresponding to 20-, 10-, 5-, and 2.5 cc. of the original culture were added to solutions of the rH indicators for a reduction test. Each one of the tubes received also 0.2 cc. of a 1 per cent glucose solution. The time required for the reduction of the dyes is given in Table VIII.

Table VIII shows: 1) that the time of reduction increases as the amount of cell decreases; 2) that the dyes are decolorized in the order of the electromotive series; 3) that all dyes tested were reduced.

TABLE IX

The Reduction of Thiol Groups by Glucose (0.2 cc. of 1 Per Cent Solution) in the Presence of Cells of Pneumococcus

Nature of the oxidized thiol compounds	Incubation period (hours)			
	24	48	72	96
Glutathione (5 cc. of meat infusion).....	±*	±	+	+
5 cc. of saturated solution of cystine.....	−*	−	±	+
1 cc. of 0.05 per cent oxidized thioglycollic acid....	+*	++*	++	++

* In this table − indicates a negative test for −SH.
± indicates a doubtful test for −SH.
+ indicates a positive test for −SH.
++ indicates a strongly positive test for −SH.

The Reduction of Thiol Compounds by the Pneumococcus-Glucose System

In view of the importance of glutathione in cellular metabolism and of the fact that thiol compounds have been found to be an essential component of the media used for the growth of Pneumococcus,[1] an attempt has been made to find out whether oxidized thiol compounds can be reduced by a system consisting of Pneumococcus and glucose.

Experiment 9.—The set up of the experiment was the same as in Experiment 8, except that meat infusion, cystine and oxidized thioglycollic acid (see Experimen-

[1] Unpublished observation.

tal Methods) were used instead of the dyes. The systems were incubated for several days, and the presence of reduced thiol groups was tested for by the nitroprusside test.

Experiment 9 brings out the interesting fact that oxidized thiol compounds are reduced by glucose in the presence of Pneumococcus cells. This reduction could be obtained with as little as 2.5 cc. of cells.

The Formation of Peroxide by Washed Cells of Pneumococcus in the Presence of Glucose and Meat Infusion

Avery and Neill (5) have conclusively shown that Pneumococcus cells, when washed in saline, lose their ability to form peroxide, but regain it in the presence of yeast extract or meat infusion. Their results also indicate that the reduction of methylene blue and the formation of peroxide are controlled by the same mechanism. It was therefore tempting to see if peroxide formation could be obtained with the same system (washed cells + sugar-free meat infusion + glucose) which proved to be able to reduce methylene blue.

Experiment 10.—Young cells of D/39/R were repeatedly washed in saline and samples taken after the first, third, and fifth washings. Amounts of cells corresponding to 25 cc. of culture were suspended in 10 cc. of buffer at pH 7.8, and glucose and sugar-free meat infusion were added as described in Table X. The mixtures were transferred to 150 cc. Erlenmeyer flasks and incubated at room temperature. A test for peroxide was made after different periods of incubation, and the results corresponding to 18 hour periods are given in Table X.

It appears from the results of Experiment 10 that washed cells of Pneumococcus can form peroxide when placed in the presence of glucose and of sugar-free meat infusion under aerobic conditions. However, this property is completely lost after the cells have been washed 5 times. Peroxide formation and methylene blue reduction therefore appear as two similar reactions, in which oxygen and methylene blue respectively act as hydrogen acceptor.

The Activity of Carbon Compounds Other than Glucose in the Presence of Washed Cells of Pneumococcus

The reduction of the indophenols, of methylene blue and of the indigoes by washed cells of Pneumococcus, has been attempted in the

presence of inulin, lactose, saccharose, maltose, dextrose, levulose, mannose, arabinose, xylose, ribose, mannite and glycerine. It would be too long to report now the results of these studies. However, it may be said that the following substances have been found to be the most active (in order of velocity of reduction): levulose, dextrose, galactose, mannose, maltose, lactose. These same substances were also found to give rise to the formation of peroxide in the presence of washed cells of Pneumococcus under aerobic conditions; the test for peroxide in

TABLE X

The Formation of Peroxide by Washed Cells of Pneumococcus in the Presence of Glucose and Sugar-free Meat Infusion

Amount of cells	No. of washings	Glucose (20 per cent solution)	Meat infusion	Peroxide
cc.		*cc.*	*cc.*	
0		0.2	0.5	−
25	1	0	0	−
25	1	0.2	0	+
25	1	0	0.5	−
25	1	0.2	0.5	++
25	3	0	0	−
25	3	0.2	0	±
25	3	0	0.5	−
25	3	0.2	0.5	+
25	5	0	0	−
25	5	0.2	0	−
25	5	0	0.5	−
25	5	0.2	0.5	−

the same system was doubtful or negative when other compounds were used which bring about reduction of the methylene blue only slowly or not at all.

DISCUSSION

An interpretation of the facts which have just been presented requires an understanding of the nature of the interreaction between the glucose and the Pneumococcus cell. Is the glucose molecule split into reducing products, or does it itself become highly reactive owing to

some molecular rearrangement? The determination of the ratio between the amounts of glucose used and of methylene blue reduced is the first step in the elucidation of this problem.

As indicated above (Experiment 7), the value of this ratio seems to be affected by a number of side reactions, but a ratio very close to 1 can be obtained under proper conditions. It may be recalled here that Quastel has already attempted to determine the value of the same ratio. He found that, at pH 7.2, a single molecule of glucose donates at least 4 (and possibly 6) atoms of hydrogen to methylene blue in the presence of *B. coli*; galactose was less active and it was impossible to state whether this sugar donates more than 2 atoms of hydrogen per molecule. The least quantity of formic acid which could accomplish complete reduction of methylene blue was the amount to be expected if formic acid donated 2 atoms of hydrogen per molecule. Now, it is perhaps reasonable to assume that *B. coli* has a wider range of activation (in Quastel's terminology) than Pneumococcus, and that many of the products which result from the splitting of the glucose molecule by the cell become able to reduce methylene blue in the presence of *B. coli*, but not in the presence of Pneumococcus.[2] This would account for the narrow glucose- methylene blue ratio obtained with the organism first mentioned.

As a result of these observations, it appears likely that the first and fundamental reaction, in the reduction of methylene blue by the Pneumococcus-glucose system is one in which one molecule of the sugar becomes able to cause the reduction of one molecule of the dye, *i.e.*, to donate two atoms of hydrogen.

Nothing is known of the mechanism of the reaction. However, the following possibilities may be considered:

1. The glucose molecule is split into two fractions, each one donating one hydrogen atom.

2. The glucose molecule undergoes some rearrangement; two of its hydrogen atoms becoming "activiated." Examples of such "activation" are not unknown; succinic acid, for instance, although unable to reduce methylene blue by itself, does it in the presence of muscle tissue

[2] It has been established for instance that succinic acid, which is an excellent hydrogen donator in the presence of *B. coli* is inactive in the presence of Pneumococcus (unpublished observation).

and *B. coli* and is oxidized to fumaric acid in the course of the process. Concerning the hypothetical "activation" of glucose, it may be mentioned here that Aubel, Wurmser, Geloso, and Genevois (8) have observed the establishment of highly reducing potentials by glucose solutions at different electrodes. It would not be without bearing on this discussion to observe the potentials obtained with sterile glucose solutions compared with glucose in the presence of Pneumococcus cells.

3. Whatever may be the nature of the reducing substances formed from the glucose, another question remains to be answered. Do the glucose derivatives reduce the methylene blue directly, or do they act first on some of the cell components which in their turn reduce the hydrogen acceptor?

A knowledge of those constituents of the meat infusion which take part in the reaction would also greatly help in an understanding of its mechanism. Meat infusion contains autoxidizable substances which are able to reduce rH indicators by themselves (6); are these substances the ones which activate the system consisting of washed cells of Pneumococcus and glucose?

This is not a problem of theoretical interest only. The utilization of the nutrients in the process of growth is probably dependent upon what Quastel calls an "activation" of these nutrients by the cell under consideration (2). Is it not possible that the failure of Pneumococcus and other fastidious organisms to grow in synthetic media is due partly to the inability of these cells to "activate" the metabolites put at their disposal? It would be interesting to determine in what measure the ability to grow in synthetic media is related to the power of "activation" in the absence of meat infusion.

Experiment 8 indicates that glucose, in the presence of Pneumococcus cells, is able to reduce rapidly all the rH indicators, including indigo disulfonate. The reduction of glutathione by the Pneumococcus-glucose system is another evidence that this system can reach very negative potentials. Too little is known as yet to appreciate the significance of this reduction; however, its importance is suggested by the fact that thiol groups are essential for the growth of Pneumococcus[3] and that the addition of reduced cysteine to the medium always increases the rate of growth.

[3] Unpublished observations.

The formation of peroxide by glucose in the presence of Pneumococcus cells affords an explanation of Cole's (4) finding in 1914 that, although washed cells of Pneumococcus do not change hemoglobin to methemoglobin in the presence of air, they do so when traces of dextrose, or of certain other substances are added to the mixture. It is interesting that the substances which exhibit this property, also form peroxide in the presence of Pneumococcus cells. Ribose and inulin which fail in this particular are inactive in both instances. Since Avery and Neill (5) have shown that peroxide and methemoglobin formation are the expressions of the same mechanism, the relation between the presence of dextrose and the oxidation of hemoglobin appears evident.

Finally, it may be proper to point out briefly the significance of these observations for the characterization of bacterial cultures by their reducing properties. The bacteriological literature offers a great number of observations concerning the "reducing power" of different species of microorganisms. It is now known that sterile media themselves develop potentials corresponding to a great intensity of reduction (6, 9, 10); Coulter (11) has recently shown that typhoid bacilli, by using up the oxygen in solution in a medium, bring about the establishment of a potential similar to that which would develop after deaeration of the same sterile medium. The experiments reported here indicate that the amount and velocity of reduction of a dye in a culture depend largely on the presence of certain metabolites. What is, then, the "reducing power" of a tissue and of a culture? Is it only an expression of its ability to activate the molecules of the substrate, or does the protoplasm possess reducing properties of its own, determined by its physical and chemical constitution?

SUMMARY

The reducing power of plain broth cultures of Pneumococcus is largely dependent upon the presence in the medium at the time when the reduction test is performed of certain metabolites.

The washed cells of Pneumococcus are able to reduce the various indicators of oxidation-reduction potentials in the presence of glucose. The relative velocity of reduction of these indicators is determined by the number of cells used in the test, the concentration of the dyes, and their position in the oxidation reduction scale.

Oxidized thiol compounds (glutathione, cystine, oxidized thioglycollic acid) are likewise rapidly reduced by glucose in the presence of washed cells of Pneumococcus.

This Pneumococcus-glucose system is able to form peroxide under aerobic conditions. Those substances which form peroxide in the presence of Pneumococcus cells are also the ones which Cole found to be active in changing hemoglobin into methemoglobin under the same conditions.

The power of washed cells of Pneumococcus to reduce methylene blue in the presence of glucose is dependent on at least 2 constituents: one which can be readily removed from the cell by washing. Sugar-free meat infusion will function instead of it. The other is inactivated more slowly by the process of washing and is destroyed by 10 minutes heating at 55°C.

The interreaction between the glucose and the cell seems to result in a fundamental reaction in which one molecule of glucose becomes able to reduce rapidly one molecule of methylene blue. The existence of side-reactions often obscures this ratio.

The significance of these observations is considered in relation to the nature and mechanism of the "activation" of metabolites, the preparation of synthetic media, the phenomena of growth, and the meaning of the expression "reducing power of a bacterial culture."

ADDENDUM

There has just appeared in the *Compt. Rend Acad. Science* an article by R. Wurmser and J. Geloso (12) in which these authors present data indicating that, under anaerobic conditions, the glucose molecule changes to a new form developing a reducing intensity corresponding to rH = 8.2 at pH 7. This form seems to give rise to a reversible system of oxidation-reduction, with an rH = 15 corresponding to a mixture of 50 per cent oxidant-50 per cent reductant. Even in the presence of oxygen, the rH of this system does not go higher than 24.

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EXPERIMENTAL PNEUMONIA IN GUINEA PIGS

II. Effect of Anti-autolysate Sera on Pneumococcus Pneumonia in Guinea Pigs

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In a previous communication (1) it was shown that extensive pneumonia associated with unrestrained multiplication of the organisms may be produced regularly in guinea pigs by the intratracheal injection of sublethal doses of certain toxic autolysates (pneumotoxin) mixed with sublethal doses of living pneumococci. Neither the pneumococci alone nor the toxic autolysate alone produced a comparable condition.

In the present paper, experiments are reported which demonstrate (a) that under certain conditions, anti-autolysate sera prepared in rabbits or horses by immunization with sterile filtrates of pneumotoxin prevent the development of pneumonia in animals inoculated with mixtures of pneumococci and autolysate; (b) that the protection against the development of pneumonia exhibited by these sera is heterologous, at least as regards Types I and II; (c) and that certain anti-pneumococcus horse sera used in the treatment of pneumonia in man contain either no heterologous pneumonia-preventing antibodies or slight amounts only.

EXPERIMENTAL

Methods

Immunization of Animals.—Rabbits were immunized over a period of 2 or 3 months by intracutaneous, subcutaneous or intravenous injections of sterile Berkefeld filtrates of the toxic autolysates from either Type I or Type II pneumococci, each rabbit being inoculated with the autolysate of one type only. The horses were immunized with subcutaneous injections of either mixed autolysate filtrates from Pneumococcus I, II and III or with mixed autolysates and the living organisms of all three types. The anti-autolysate sera from the horses were concentrated and refined by the usual methods and in this form tested for their

pneumonia-preventing properties. Normal rabbit or normal horse sera were used as serum controls in the work.

The pneumococcus strains—Types I and II—were the same as those used in our previous work. A strain of Pneumococcus III was also used. This was isolated from a patient with pneumonia in the Presbyterian Hospital in May, 1928. The method of preparing the toxic autolysates has been described in previous papers (1, 2). Only those filtrates from Types I, II and III were used which would kill guinea pigs within 24 hours in doses of 0.2 cc. when injected intratracheally.

Young guinea pigs weighing from 190 to 250 gm. were used, and owing to the fact that the susceptibility to the autolysate usually varies inversely with the weight of the pig, it was found important to have the animal in each experiment as nearly the same weight as possible.

The injections of serum were given intraperitoneally 14 to 24 hours before the intratracheal infecting dose of pneumococci and autolysate. At first, the rabbit immune sera were given intraperitoneally 24 hours previous to the intratracheal dose of organisms and pneumotoxin, but some of these sera caused the development of anaphylactoid reactions immediately after the intratracheal inoculations. In later experiments the serum injections were made 14 to 18 hours before the intratracheal dose and in this way the anaphylactoid reactions were avoided. Our anti-pneumotoxic horse sera have never given anaphylactic sensitization, even when the sera were given 24 hours before the infecting dose of toxin and pneumococcus (3).

Intratracheal Injections.—In carrying out the pneumonia preventing tests, the proper adjustment of the doses of pneumotoxin and pneumococcus is essential. The dose of pneumococcus used was about one half that which would cause death from septicemia when injected intratracheally without mixture with autolysate. The same principle governs the dose of autolysate used in these experiments. The amount employed was one half the quantity necessary to kill a pig of approximately the same weight in 24 hours. With experience as to the virulence of the culture and the strength of the autolysate, it is comparatively easy to obtain the proper amounts of autolysate and pneumococci for these experiments.

The intratracheal injections were carried out as follows:—An 18–24 hour broth culture of the pneumococcus was diluted with broth to the strength desired. This tube, containing the diluted pneumococcus culture, was kept in ice water throughout the experiment.

A tube containing the toxic filtrate was chilled, the vaseline seal opened, and the filtrate pipetted into a narrow, chilled tube. This tube, containing the toxic autolysate, was also kept in ice water throughout the experiment. Just before the injection, 0.2 cc. of the diluted pneumococcus culture and 0.2 cc. of the autolysate were pipetted into another iced tube, mixed, and 0.2 cc. of the mixture was drawn into an iced syringe and immediately injected intratracheally into a guinea pig. A fresh mixture was always prepared for each injection and the same precautions as to chilling, etc., were carried out with both control preparations consisting of pneumococcus and broth, and autolysate and broth.

Protective Action of Anti-autolysate Sera Against the Pneumonia Caused by Pneumococci Plus Toxic Filtrates of Homologous Type

Table I demonstrates the protective action of two preparations of concentrated anti-autolysate horse sera against pneumonia caused by mixtures of toxic autolysate Type I and living pneumococcus Type I.

This experiment shows that two pigs injected intraperitoneally with 1 cc. each of normal horse serum 18 hours previous to the inoculation of

TABLE I

Action of Anti-autolysate Sera in the Prevention of the Pneumonia Caused by Pneumococci and Toxic Filtrates of Homologous Type

Pneumococcus culture Type I; very slight growth. Autolysate from Type I. 0.2 cc. pneumococcus, Type I culture + 0.2 cc. autolysate. 0.2 cc. inoculated intratracheally; Concentrated autolysate horse 30336. 9/24/28. Concentrated autolysate horse 30337. Normal horse serum.

No.	Wt.	Intraperitoneal injection 10/1	Intratracheal injection 10/2	Symptoms	Died or survived	Cultures		Extent of lung consolidation
						Heart	Lung	
55	238	1 cc. normal horse serum	Pn. + autolysate	+++	D. 3 days	+++	+++	++
58	230	1 cc. 30336	Pn. + autolysate	0	S.	−	−	−
69	232	1 cc. 30337	Pn. + autolysate	0	S.	−	−	−
61	226	—	Broth + autolysate	+	S.	−	−	−
64	224	—	Pn. + broth	0	S.	−	−	−
54	230	1 cc. normal horse serum	Pn. + autolysate	+++	D. 2 days	+++	+++	+++
56	232	1 cc. 30336	Pn. + autolysate	0	S.	−	−	−
65	238	1 cc. 30337	Pn. + autolysate	0	S.	−	−	−
57	222	—	Broth + autolysate	+++	S.	−	−	−
60	222	—	Pn. + broth	0	S.	−	−	−

pneumococci and toxic autolysate died in 2 and 3 days respectively, with pneumonia and septicemia; while four pigs previously prepared with 1 cc. each of two preparations of concentrated anti-autolysate sera remained well. The difference in the appearance of the protected and unprotected animals was striking. 18 hours after the intratracheal injection of pneumococcus and autolysate the two pigs which had received the normal serum were severely dyspnoeic and appeared extremely sick, while the pigs which had received the anti-autolysate

serum had normal respiration and appeared well. The two pigs which were injected with the autolysate and broth were dyspnoeic, one being desperately sick for 8 days but finally recovering; while the two which were injected with the pneumococcus broth culture remained well.

Serum preparation 30336 was obtained from two horses immunized with toxic autolysate filtrates and contained no specific protective substances. Serum preparation 30337 was obtained from two horses immunized with toxic autolysates and living pneumococci and protected Pn. $1,10^{-4}$. The question of the importance of pneumococcus specific protective properties in these experiments will be considered later.

Protective Action of Anti-autolysate Sera on the Pneumonia Caused by Pneumococci Plus Toxic Filtrates of a Heterologous Type

That the pneumonia preventing action of our anti-autolysate sera is heterologous as regards pneumococcus Types I and II has been demonstrated conclusively many times. These experiments were carried out with anti-autolysate sera produced in rabbits by the immunization with autolysates of one type only (Type I or Type II). The experimental pneumonia in each instance was caused by the autolysate and pneumococci of heterologous strains (Type I or II). Table II demonstrates the preventive action of Type I anti-autolysate serum against pneumonia caused by pneumococcus and autolysate Type II, and is self explanatory. The same pneumonia-preventing action has been demonstrated for rabbit anti-autolysate Type II sera against the pneumonia caused by injections of Pneumococcus I toxin and living pneumococcus I.

These experiments therefore demonstrate that our anti-pneumotoxic sera are definitely protective against pneumonias caused by mixtures of pneumococci and autolysates of a heterologous strain.

Action of the Anti-pneumococcus Sera Used in the Treatment of Human Pneumonia for the Pneumonia-preventing Heterologous Substances

In testing the anti-bacterial pneumococcus sera for the presence of the pneumonia-preventing substances which have been shown to be present in our anti-autolysate sera, it was important to rule out the

effect of the specific protective substances which all these anti-bacterial sera contain. To accomplish this, it was only necessary to test out the efficacy of these sera against the pneumonia produced by a pneumococcus and autolysate from a heterologous strain.

Table III gives one of our experiments of this kind. It is seen that the serum concentrated by the Felton method shows no heterologous pneumonia-protective powers; the serum produced by the Zinsser

TABLE II

Action of an Anti-autolysate Serum in Preventing Pneumonia Caused by Pneumococci Plus Toxic Filtrates of a Heterologous Type

Pneumococcus II—good growth. Diluted 1–10 with broth Autolysate II. 0.3 cc. Pn. II dilution + 0.1 cc. autolysate. 0.2 cc. mixture injected. Controls: (a) 0.3 cc. Pn. + 0.1 cc. broth—0.2 cc. injected. (b) 0.3 cc. broth + 0.1 cc. autolysate—0.2 cc. injected. Rabbit serum 7–54, Anti-autolysate I.

No.	Wt.	Intraperitoneal injection 5/9	Intratracheal injection 5/10	Symptoms	Died or survived	Cultures		Extent of lung consolidation
						Heart	Lung	
10-11	204	2 cc. normal rab.	Pneumococcus + autolysate	+++	D. 40 hrs.	+++	+++	+++
8-74	202	2 cc. 7–54	Pneumococcus + autolysate	0	S.	–	–	–
8-88	204	2 cc. 7–54	Pneumococcus + autolysate	0	S.	–	–	–
8-89	210	—	Pneumococcus + autolysate	+++	D. 40 hrs.	+++	+++	+++
10-09	206	—	Pneumococcus + autolysate	+++	D. 18 hrs.	+	+++	+++
8-87	200	—	Broth + autolysate	0	S.	–	–	–
10-06	206	—	Pneumococcus and broth	0	S.	–	–	–

method shows a slight amount (protected one of the two pigs injected), while the rabbit anti-autolysate serum gives perfect protection. The same results were obtained in another experiment carried out in a similar way. Still another anti-pneumococcus serum used in treating human pneumonia was tested for heterologous pneumonia-preventing substances, and proved to be even weaker in these properties than the serum produced by the Zinsser method.

These experiments demonstrate how poor in heterologous anti-pneumonia antibodies the ordinary anti-bacterial pneumococcus sera are. The explanation for the complete absence of these antibodies in the serum produced by the Felton method is probably to be found in its method of preparation, which concentrates the specific protective

TABLE III

Action of Certain Anti-pneumococcus Sera Used in the Treatment of Human Pneumonia in Preventing the Pneumonia Caused by Autolysate and Pneumococci of a Heterologous Type

Pneumococcus Type II; slight growth on broth. Autolysate Type II. 0.2 cc. Pn. culture + 0.2 cc. autolysate. 0.2 cc. injected. Controls: Pn. + broth and autolysate and broth. Sera: Felton's antibodies I; Zinsser anti-pneumococcus serum; Rabbit 7–54; Anti-autolysate I.

No.	Wt.	Intraperitoneal injection 3/2	Intratracheal injection 3/3	Symptoms	Died or survived	Cultures		Extent of lung consolidation
						Heart	Lung	
7-59	200	2 cc. Felton antibodies	Pn. + autolysate	+++	D. 2 hrs.	0	+	++
7-77	200	Same	Pn. + autolysate	+++	D. 2 days	++	+++	+++
7-67	200	2 cc. Zinsser serum	Pn. + autolysate	0	S.	−	−	−
7-78	205	Same	Pn. + autolysate	+++	D. 40 hrs.	++	+++	+++
7-73	200	2 cc. 7–54	Pn. + autolysate	++	S.	−	−	−
7-66	200	2 cc. 7–54	Pn. + autolysate	+	S.	−	−	−
7-69	195	2 cc. normal horse	Pn. + autolysate	+++	D. 18 hrs.	+++	+++	+++
7-75	200	2 cc. normal rabbit	Pn. + autolysate	+++	D. 5 hrs.	0	+	++
7-71	190	—	Pn. + autolysate	+++	D. 40 hrs.	+++	+++	+++
7-80	195	—	Broth + autolysate	+	S.	−	−	−
7-74	190	—	Pn. + broth	0	S.	−	−	−

bodies in the globulin fraction of the serum and discards that part of the serum which contains neutralizing antibodies (the pseudoglobulin fraction.)

It is logical to suppose that a serum containing specific protective antibodies, would have a preventive effect by its antibacterial properties, on the pneumonia caused by the homologous type of organism and autolysate; and that this preventive effect would correpond closely to

the amounts of specific protective substances which these sera contain. This was found to be the case. The serum produced by the Felton method, containing 1000 protective units, protected 75 per cent of the animals injected; the Zinsser serum protected 50 per cent, and the other Anti-pneumococcus I serum only delayed death in one instance. To save space, a table giving the details of these experiments is omitted.

Protective Effect of Normal Rabbit or Horse Serum

Occasionally normal rabbit serum protects against the pneumonia produced by a mixture of living pneumococcus and autolysate. We believe this to be due to a non-specific reaction induced by the foreign serum in a small percentage of the injected pigs (about 8 per cent). Horse serum occasionally shows this non-specific protective action. Stillman had the same experience in his pneumococcus work in mice. He found that normal rabbit serum injected intraperitoneally in mice sometimes protected them from pneumococcus infections. These rabbit sera contained no pneumococcus specific protective substances (4).

The Content of Pneumococcus Specific Protective Substances in the Anti-autolysate Sera

That the pneumonia-preventing properties of anti-autolysate sera are not related to the pneumococcus specific protective substances was demonstrated conclusively by the fact that the anti-autolysate sera are equally effective against a pneumonia due to the toxin and pneumococci of either a heterologous or homologous strain. We also titrated all our rabbit anti-autolysate sera and in no instance did they show the presence of specific protective substances for mice. Neither were there specific protective substances in horses immunized with the autolysate filtrates, but protective substances for pneumococci Types I and II were present in small amounts in the sera obtained from the horses which had been injected with living pneumococcus Types I, II and III, in addition to the autolysates.

DISCUSSION

The experiments reported in this paper appear to indicate that under certain conditions the sera produced in rabbits or horses by immuniza-

tion with Berkefeld filtrates of certain anaerobically produced autolysates of pneumococcus Type I or II protect against the pneumonia caused in guinea pigs by the intratracheal injection of mixtures of living pneumococci and toxic autolysates. Since the sera contain no specific anti-bacterial antibodies, it is evident that their protective action must be due to some other form of immune body. The immune body present differs also from the specific anti-bacterial bodies in that it is apparently identical for both Types I and II of the pneumococci and is therefore not type specific.

Whether this lung-toxic substance is produced in spontaneous pneumonia, or in the experimental pneumonia produced by intratracheal injections of pneumococci alone—as in the dog or monkey—is a question which cannot be answered at present. The demonstration that a toxic substance of this nature is present in these conditions would help to explain some of the obscure features of pneumococcus pneumonia in animals and man.

In the production of curative sera for lobar pneumonia, most of the efforts have been devoted to obtaining sera with a high content of specific protective antibodies against living organisms, but Cecil and Blake (5) have found that the serum of a monkey may be completely free of any specific protective substance and yet be highly immune to pneumonia. The reverse was also found to occur. A monkey whose serum may protect mice against 100 to 1000 M.L.D.'s of pneumococci may still be susceptible to the homologous pneumococcus pneumonia.

In connection with this work, it is also of interest to note that Cecil and Blake demonstrated that resistance to experimental pneumonia in monkeys is to some degree species specific as regards pneumococci Types I, II and III (6). Stillman (7) has come to the same conclusion in his work on the production of pneumococcus pneumonia in mice by the inhalation method. He found that mice which had been rendered immune to one type of pneumococcus (Type I) by the inhalation method, may show an increased resistance locally when exposed to infection by the same method with organisms of another type (Type II).

CONCLUSIONS

1. Anti-pneumotoxic sera prepared in rabbits or horses by immunization with sterile filtrates of the pneumotoxin, under certain condi-

tions protect against the pneumonia caused by the intratracheal injections of mixtures of living pneumococci and toxic autolysates.

2. The protection against the development of pneumonia is heterologous, at least as regards Type I, Type II, *viz.*: an anti-autolysate serum prepared by the immunization with a pneumotoxin from one type of pneumococcus will prevent the development of pneumonia caused by the injection of pneumococci and autolysate from another type.

3. Certain anti-pneumococcus horse sera used in the treatment of pneumonia in man, either contain no heterologous pneumonia-preventing antibodies or slight amounts only. These sera, however, protect against the pneumonia produced by injections of pneumococci and pneumotoxin of the homologous strain, the degree of protection depending on the amount of specific protective substances such sera contain.

4. Anti-pneumotoxic sera produced in rabbits or horses by the injection of sterile Berkefeld filtrates of the toxic autolysates contain no pneumococcus specific protective substances.

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STUDIES ON INFLAMMATION

I. Fixation of Vital Dyes in Inflamed Areas

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Inflammation involves local physiological changes in the permeability of capillaries, in the rate of blood flow, and perhaps in the normal balance of body fluids. Increase of capillary permeability is shown by local edema. With the passage of proteins from the plasma into the inflamed area the osmotic relationship between tissue fluids and blood plasma is doubtless disturbed. About 30 years ago Adler and Meltzer (1) stated that the passage of fluids from tissue spaces into lymphatics probably depends on osmosis perhaps assisted to some extent by filtration. Krogh (2) recently pointed out that the passage of proteins from capillaries increases the osmotic pressure of the tissue fluids. This change in an inflamed area would probably modify the passage of fluid into the regional lymphatics which drain the inflamed area.

The dissemination of foreign substances from the site of injection into the lymphatics and blood stream has been studied by various investigators. Muscatello (3) showed that carmine and various other inert particles when injected into the peritoneal cavity reach the anterior mediastinal lymph nodes very rapidly. Noetzel (4) injected bacteria (*B. pyocyaneus*) into the knee joints of rabbits. 5 to 10 minutes later he was able to demonstrate the presence of the organisms in the inguinal, crural, and lumbar lymph glands. Buxton (5) found that typhoid bacilli when injected into the peritoneal cavity, appeared in great numbers within a few minutes in the blood stream. Wells and Johnstone (6) showed that the absorption of bacteria from the peritoneal cavity takes place through lymphatic vessels.

Within recent years evidence has accumulated to show that an inflammatory reaction at the site of injection might be a factor in delaying the passage of foreign substances into the blood stream. Pawlowsky (7) demonstrated that the dissemination into the blood stream of staphylococci injected into a previously inflamed knee joint was either inhibited or wholly prevented. The studies of Issayeff had showed that peritonitis caused by a sterile irritant increased temporarily the resistance of the animal to subsequent intraperitoneal inoculation of bacteria (8). Opie (9, 10) showed that foreign protein injected into the dermis of an immunized animal was fixed at the site of injection; the contact of antigen and

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antibody at the site of injection causes an acute inflammatory reaction (Arthus phenomenon). In normal animals, on the contrary, foreign proteins when injected into the dermis caused little if any inflammatory reaction and readily penetrated into the blood stream. Willis (11) studied the dissemination of tubercle bacilli from the site of cutaneous inoculation and showed that in reinfected guinea pigs the spread of tubercle bacilli from the site of inoculation is retarded, whereas in normal animals the organisms pass readily to the regional lymph nodes. Opie (12) recently showed that acute inflammation of the peritoneal cavity caused by aleuronat retards the rush of injected hemolytic streptococci from the peritoneal cavity into the circulating blood and after 24 hours completely prevents it.

The present study was undertaken in order to determine whether various foreign substances passed to tributary lymphatic vessels when injected into an inflamed area. At first, substances consisting of large particles were used. Nucleated cells from fowl's blood were unsuitable for the experiment because they failed to pass into the regional lymphatics when injected into normal subcutaneous tissue. *Bacillus subtilis* was tried with the same negative results. It was in the course of these preliminary observations that trypan blue was tried. This vital dye when injected into the subcutaneous tissue of the extensor surface of the fore-leg of a rabbit appeared within 30 to 40 minutes in the lymphatic vessels of the axilla (13). The tributary afferent lymphatic vessels, the lymph node, and the efferent lymphatic vessel revealed plainly the presence of the dye. If, however, the dye was injected into an area in which an inflammatory reaction had been produced some time before, the dye did not appear in the tributary lymphatics. The afferent lymphatics, the lymph node, and the efferent lymphatic vessel remained colorless. The dye was evidently retained in the inflamed area.

The Retention of Trypan Blue in Areas of Inflammation

Sterile inflammation was produced by a mixture of 5 per cent of aleuronat and 3 per cent of starch in 0.5 per cent saline solution. Usually 5 cc. of this solution were injected into the subcutaneous tissue of the extensor surface of the fore-leg of a rabbit 2 or 3 cm. from the shoulder joint. Either immediately following the injection of aleuronat or after varying intervals of time, 3 cc. of a 1 per cent solution of trypan blue in saline were injected subcutaneously into the inflamed area. The same amount of dye was injected into the other fore-leg to serve as a control. The lymph from the efferent lymphatic vessel was collected as recently described by Freund and Whitney (14). To expose the lymphatic the pectoral muscles were

incised and the subclavian vein exposed, under ether anesthesia. Alongside and somewhat below the vein an efferent lymphatic vessel was seen to emerge from one of the axillary lymph nodes. The lymph was drawn by puncturing the efferent lymphatic and introducing a capillary pipette into it. On the normal side the lymph thus obtained from the efferent lymphatic was stained with the dye. Varying concentration of trypan blue in the lymph was more conspicuous when the capillary

TABLE I

Retention of Trypan Blue at the Site of Inflammation

Rabbit No.	Interval between injection of irritant and that of dye	Duration of inflammation	Presence of dye on normal side		Presence of dye on inflamed side	
			Lymph of efferent lymphatic	Lymph node	Lymph of efferent lymphatic	Lymph node
	hrs.:mins.	*hrs.:mins.*				
1	0:00	1:30	++	++	trace	trace
2	0:00	2:00	+	+	+	+
3	0:00	2:20		trace		faint trace
4	0:30	3:30	+	+	0	0
5	1:00	3:40	++	++	0	0
6	2:10	4:30	+	+	0	0
7	4:00	6:00	+	+	0	0
8	19:00	20:30	++	++	0	0
9	19:30	20:30	++	++	0	0
10	20:30	23:00	+	+	faint trace	0
11	22:00	23:00	++	++	0	faint trace
12	22:30	23:00	+		0	0
13	22:30	23:30	+	+	0	0
14	23:30	28:30	+	+	0	0
15	24:00	25:30	+	+	faint trace	trace
16	25:00	26:00	++	++	faint trace	trace
17	25:00	26:00	faint trace		0	
18	26:00	27:00	++	++	trace	+
19	46:00	47:30	+++	+++	0	0
20	46:15	48:00	+	++	trace	0
21	46:15	48:15	+	++	0	faint trace

pipettes containing lymph were held against a white background. The axillary lymph nodes were exposed and removed in order to determine if they were stained by the dye.

The results of this series of experiments appear in Table I. A colorless lymph is indicated by zero; a distinctly blue color by one plus (+); a dark blue color by two plus signs (++); and a bluish-

black color by three plus signs (+++). "Trace" or "faint trace" of blue refers to barely perceptible staining of lymph or lymph nodes.

When trypan blue was injected in both fore-legs of the animal immediately after the inflammatory irritant, the passage of the dye occurred in both the normal and inflamed side with about equal intensity, but in Rabbit 1 the concentration of the dye was less on the inflamed side. If, however, the dye was injected only 30 minutes after the irritant (Rabbit 4), trypan blue did not pass either into the lymph node or its efferent lymphatic vessel on the inflamed side. Evidently the fixation or retention of the dye in an inflamed area occurs very early and is seen at least 30 minutes after the onset of inflammation. The fixation of the dye at the site of inflammation was proven not merely by the absence of pigment in the tributary lymph node but by its failure to appear in the lymph from the afferent lymphatic vessels on the inflamed side and its presence on the normal side in four rabbits. Lymph was in most instances obtained 1 to 2 hours after the injection of the dye. In one animal (Rabbit 14) in which the lymph was collected after 5 hours the result was the same, *i.e.*, fixation of the dye in the inflamed area.

The Accumulation of Trypan Blue in Inflamed Areas

Since trypan blue tends to remain *in situ* when injected directly into the inflamed area, the attempt was made, at the suggestion of Dr. Eugene L. Opie, to determine whether the dye injected into the blood stream would accumulate at the site of inflammation.

A few years ago Lewis (15) found that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. 24 hours or more after such an inoculation, if the animal is injected intravenously with trypan red, the fluid in the anterior chamber of the inoculated eye always becomes colored. He also found that an intense conjunctivitis occurs when abrin is dropped in the eye. Here again, when trypan red or trypan blue is injected intravenously at different stages of this inflammation, the dye appears rapidly in the anterior chamber and in the edematous conjunctiva. Ramsdell (16) injected trypan blue into the veins of rabbits and guinea pigs previously treated with foreign serum and found that injection of the same serum into the skin of the ear, immediately caused local infiltration of the dye into the adjacent tissue. She regarded this infiltration of

the dye as an indicator of edematous changes resulting from toxic injury to the capillary endothelium.

Okuneff (17) found that the application of a thermal irritant favors the passage of vital stains from the blood stream into the area heated. Kusnetzowsky (18) also observed that the local application to the skin of an irritant such as heat or mustard oil causes an accumulation of trypan blue in the inflamed area when the dye has been previously injected into the blood stream. He found the dye to be concentrated both in the cells of the connective tissue and especially in the histiocytes.

An inflammation was produced by injection of aleuronat into the subcutaneous tissue of the fore-leg of a rabbit. A 1 per cent solution of trypan blue was injected intravenously in varying quantities ranging from 5 to 10 cc. In three rabbits the dye was injected intravenously immediately after injection of the inflammatory irritant into the fore-leg. In the remaining experiments the dye was injected at various intervals of time after the inflammatory irritant. When the inflammatory reaction had proceeded for varying periods of time, as shown in Table II, the area of inflammation and the tributary lymph node were exposed and compared with the corresponding normal areas on the opposite fore-leg to determine the presence of trypan blue. Even when the dye was injected intravenously immediately after the local injection of irritant, there was a greater accumulation of trypan blue in the inflamed area than in the corresponding normal area. On the inflamed side, not merely the deep fascia was more intensely stained by the dye but the muscle tissue as well. The time element in the appearance of the dye in the lymph node on the inflamed side was noteworthy. When the dye was injected immediately after the irritant, the dye appeared in the lymph node in large amounts; but if the dye was injected only $1\frac{1}{2}$ hours after the inflammatory irritant (Rabbit 25, Table II), less of it seemed to reach the lymph node of the inflamed than that of the normal side. The longer the interval of time between the injection of the irritant and that of the dye, the more efficient appeared to be the retention of trypan blue by the inflamed area, and consequently less of the dye diffused to the tributary lymph node.

A series of experiments was performed in which the inflammatory irritant used was broth (concentrated to 1/14 of its original volume) and injected into the dermis of the abdomen. The dosages ranged

from 0.1 cc. to 0.5 cc. After varying intervals of time, 5 to 10 cc. of 1 per cent trypan blue were injected intravenously. In some rabbits the dye was injected immediately after the intracutaneous injection of broth. Within usually less than 1 hour after introduction of the dye the small inflamed areas of the dermis appeared intensely blue and generally stood out prominently on the surface of the abdomen. A few experiments were repeated with bacteria acting as the inflammatory irritant. 0.1 cc. of a saline suspension of *Staphylococcus aureus* was injected into the dermis of the abdomen followed a few hours later by intravenous injection of trypan blue. Within a relatively short time the tissues in the site of inflammation appeared markedly stained. In many cases these areas showed a pale reddish central zone surrounded by an intensely dark blue band measuring about 0.5 cm. in width. The localization of intravenously injected trypan blue in inflamed areas of the dermis was evident in 32 out of 36 experiments.

TABLE II

Accumulation of Trypan Blue in Inflammatory Areas

Rabbit No.	Interval of time between injection of irritant and that of dye	Duration of inflammation	Presence of dye on normal side		Presence of dye on inflamed side	
			Normal area corresponding to area of inflammation	Lymph node	Inflamed area	Lymph node
	hrs.:mins.	*hrs.:mins.*				
22	0:00	1:15	trace	+	++	trace
23	0:00	3:20	trace	++	+++	++
24	0:00	19:00	+	trace	+++	++
25	1:30	24:00	trace	+++	++	+
26	2:30	28:00	trace	++	++	+
27	4:00	6:30	faint trace	0	+++	0
28	4:30	6:30	trace	trace	+++	faint trace
29	4:30	24:00	+		+++	+
30	5:45	7:00	0	faint trace	+++	faint trace
31	19:00	20:00	trace	++	+++	faint trace
32	20:45	21:30	+	faint trace	+++	trace
33	21:05	22:00	faint trace	0	+++	0
34	24:00	47:00	0		+	0
35	45:45	47:30	faint trace	++	++	faint trace
36	45:50	47:00	faint trace	trace	++	trace

Rabbit	Duration of inflammation	No. of inflamed areas on abdomen	No. of experiments in which inflamed areas were stained blue by the fixation of trypan blue
	hrs.:mins.		
33	0:38	4	4
31	0:45	2	2
22	1:15	4	4
37	1:00 to 3:30	6	6
26	1:30 to 4:30	5	3
25	3:00	3	3
38	4:00	4	4
34 (aleuronat)	6:30	5	4
39	21:05	3	2

The central zone which has a red congested appearance and which is often seen in these intracutaneous areas of inflammation is perhaps due to thrombosis of small vessels. Histological sections of such areas reveal some thrombosed vessels with acute inflammatory changes in the surrounding tissue.

The Fixation of Trypan Blue by an Inflammatory Reaction in the Peritoneal Cavity

It is well known that the retrosternal lymph nodes of the anterior mediastinum drain the peritoneal cavity. Years ago Muscatello (3) pointed out that carmine and various inert particles injected intraperitoneally into dogs reach the anterior mediastinal lymph nodes very rapidly. Okuneff (19) recently pointed out that certain substances injected intraperitoneally such as animal charcoal, casein or gelatine delay the absorption into the blood stream of trypan blue when the dye is injected in the abdominal cavity either simultaneously or within thirty minutes after the injection of these substances. The mechanism involved was thought to be an adsorption of the dye by these substances. As already mentioned Opie (12) has recently reported experiments in which hemolytic streptococci, injected into the peritoneal cavity of rabbits, appeared within 10 minutes in the blood stream. If 24 hours prior to the injection of these bacteria a sterile inflammatory irritant, such as aleuronat, had been injected into

the peritoneal cavity, the organisms were prevented from reaching the blood stream. In view of this interesting study, trypan blue was injected into the peritoneal cavity of normal rabbits and into that of animals which had previously received an intraperitoneal injection of aleuronat.

About a half hour after the injection of the dye into the peritoneal cavity of normal rabbits, the pigment was found in the lymph of the retrosternal lymphatics. The retrosternal lymph nodes also appeared

TABLE III

Retention of Trypan Blue by Inflammation in the Peritoneal Cavity

Experiment	Interval of time between injection of irritant and that of dye	Duration of inflammation	Presence of dye in the retrosternal lymph nodes	
			Injected trypan blue controls	Animals with peritoneal inflammation
	hrs.: mins.	*hrs.: mins.*		
1	0:00	0:34	++	++
2	2:00	2:33	++	+
3	5:08	6:00	++	0
4	22:45	28:00	+++	trace
5 (Neutral red)	23:00	24:00	++	0
6	23:10	49:00	+++	++
7	24:00	25:40	+++	trace to +
8	24:00	29:00	+++	trace
9	27:30	29:20	+++	faint trace
10	27:55	30:00	+++	+

deeply stained with the dye. The peritoneal cavity was stained only moderately. However, when trypan blue was injected into the inflamed peritoneal cavity, the retrosternal lymphatics and the retrosternal lymph nodes either failed to show the presence of the dye or occasionally showed it only in traces. The peritoneal cavity was much more deeply stained than in normal animals. Clumps or precipitates of the dye were conspicuous on the omentum and on the peritoneal surface of the diaphragm.

The amount of aleuronat used was either 5 cc. or 10 cc. The dye was injected immediately after the irritant or else after varying intervals of time. Usually 5 cc. of 1 per cent trypan blue were injected

intraperitoneally. After varying periods of time the retrosternal lymph nodes of normal and injected animals were examined for the presence of the dye. The results are shown in Table III. When the dye was injected immediately after the irritant, the retrosternal lymph nodes of normal animals and of those with an inflamed peritoneal cavity were equally colored by the dye (Experiment 1). If, however, the inflammation had been in progress as long as 2 hours when the dye was injected, the appearance of trypan blue in the retrosternal lymph nodes was conspicuously less than in the lymph nodes of the control animal. The dye is evidently prevented from leaving the peritoneal cavity by the inflammatory reaction. This fixation of dye is analogous to that of hemolytic streptococci observed by Opie (12).

CONCLUSIONS

Trypan blue injected into normal subcutaneous tissue passes rapidly to the regional lymphatic node and is found in lymph drawn from its efferent lymphatic.

When the dye is injected into the normal peritoneal cavity it rapidly appears in the lymph of the retrosternal lymphatics and stains deeply the retrosternal lymphatic nodes.

Trypan blue injected into the site of inflammation in the subcutaneous tissue or in the peritoneal cavity is fixed in the inflamed area and fails to reach the regional lymphatic nodes.

If an inflammatory reaction has been produced in the dermis or in the subcutaneous tissue, trypan blue injected into the circulating blood enters the site of inflammation and is fixed so that the tissues are deeply stained.

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OBSERVATIONS CONCERNING THE PERSISTENCE OF LIVING CELLS IN MAITLAND'S MEDIUM FOR THE CULTIVATION OF VACCINE VIRUS

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PLATE 7

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The fact that vaccine virus is capable of multiplying in cultures of susceptible tissues is well established. Some investigators (1), however, have claimed that it is possible to obtain an increase of this infectious agent in the absence of living host cells. These claims have not been substantiated. Indeed, Harde (Steinhardt) (2, 3) and Nye and Parker (4) have reported that tissues killed by freezing and thawing and by hypotonic salt solutions did not support the survival or multiplication of vaccine virus.

Recently Maitland and Maitland (5) recorded observations concerning "cultivation of vaccinia virus without tissue culture." Their medium consisted of minced chicken kidney suspended in a mixture of chicken serum (1 part) and Tyrode's solution (2 parts). These workers found that the virus increased in the absence of any detectable growth of cells in the cultures. In fact, they state that "after 24 hours the small pieces of kidney had begun to disintegrate, and by the third day autolysis of the tissue was extensive." There is no reason to doubt that vaccine virus multiplied in Maitland's medium. Moreover, Eagles and McClean (6) and Andrewes (7) have shown that certain viruses are capable of increasing under such conditions. Inasmuch as most workers have been unable to cultivate vaccine virus in the absence of living cells, and since it is known that certain cells remain viable under many conditions (8, 9), there are reasons for ascertaining whether living cells can persist or whether growth of cells can occur in Maitland's medium. It is with this problem that the present communication is chiefly concerned.

EXPERIMENTAL

Methods and Materials

Vaccine Virus.—Levaditi's neurovaccine was injected in the testicles of a rabbit. 4 days later the testicles were removed[1] and ground in a mortar with sand and M/50 phosphate solution, pH 7.6. The emulsion was thoroughly centrifuged and the supernatant fluid was used as an inoculum. The titer of the virus was determined in rabbits by means of intradermal inoculations of 0.2 cc. of serial dilutions of virus emulsions.

Culture Medium.—Throughout this work Maitland's technique of preparing and testing cultures was followed.

> "Blood was withdrawn from a hen and after it had clotted the serum was collected The hen was killed with chloroform and ether, and the kidneys, which were removed aseptically, were minced finely with scissors. Into a flask was put 0.66 c. cm. (approximately) of minced kidney and 1.33 c. cm. of inoculum diluted 1 in 6.6 with Tyrode's solution. The mixture was allowed to stand in the cold room for four hours. Then were added 12 c. cm. of Tyrode's solution and 6 c. cm. of hen's serum. The final dilution of the inoculum was thus 1 in 100. The mixture was distributed in 2 c. cm. into Carrel's tissue culture flasks, type D, which were incubated aerobically at 37°C., without caps. The cultures were tested after various periods of incubation by grinding the whole of the contents of each flask with sand and centrifugalising."

Controls.—In addition to the normal kidney tissue, controls with minced kidney tissue that had been frozen (CO_2 snow) and thawed 10 times were employed. To determine whether the tissues were living or dead at the beginning of each experiment, just prior to the distribution of the cultures in flasks, bits of the normal and of the frozen kidney tissue were placed in hen plasma and embryo extract on mica coverslips which were inverted and sealed over hollow ground slides. These preparations were then incubated at 37°C. After 5 or 6 days they were examined for evidences of cell growth.

To ascertain whether living cells persisted in the cultures prepared and handled according to Maitland's method, bits of tissue were removed from the Carrel flasks at 3, 4, and 5-day intervals, washed in Ringer's solution, and planted in plasma and embryo extract on mica coverslips These preparations were examined frequently for evidences of cell growth.

Inasmuch as it seemed possible that certain cells might at times multiply in a mixture of serum and Tyrode's solution, bits of normal kidney tissue were placed in such a mixture on mica coverslips over which hollow ground slides were placed and sealed. The slides were not inverted, since it is well known that in a liquid medium cells usually require a surface along which to grow. After incubation at 37°C., these preparations were examined for evidences of cell migration or cell growth.

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[1] All operations were performed under ether anesthesia.

To test the viability of bacteria one usually resorts to subcultures rather than to examinations of fresh and stained specimens. Consequently, in determining whether cells are able to remain alive in the medium employed by Maitland for the cultivation of vaccine virus, subcultures, as described above, of bits of tissue in a favorable medium were made. Under these conditions, it is appreciated that positive results are more significant than negative ones. In this work 4 experiments were performed, 3 of which will be described in detail. The fourth will be omitted, since the results were similar to those of the others.

Experiment I

Jan. 15, 1929.—Fresh hen kidney was minced. One portion was frozen (CO_2 snow) and thawed 10 times. Then both portions were placed in contact with vaccine virus in the ice box for 4 hours. After the exposure to virus, 10 pieces of unfrozen and frozen tissue respectively were cultured as controls in plasma and embryo extract on mica coverslips. The inoculated frozen and unfrozen tissues were then added to a mixture of serum and Tyrode's solution and distributed in Carrel flasks (2 cc. each)—3 with unfrozen and frozen tissue respectively. Titer of vaccine virus in the cultures at this time: frozen = 1:100; normal or unfrozen = 1:100.

Jan. 17.—Each of the 10 control cultures made on coverslips from the unfrozen bits of tissue showed growth of cells, while in those made from frozen tissue no evidence of cell growth was observed at this time or upon subsequent examinations.

Jan. 18.—The cultures were removed from the Carrel flasks. Bits of tissue from 2 of the flasks with unfrozen kidney and from 2 of the containers with frozen kidney were washed in Ringer's solution and subcultured in hen plasma and embryo extract. None of the subcultures of frozen tissue showed growth in 6 days (Fig. 2), while in all of those made from unfrozen tissue growth of cells (Fig.1) was evident. The predominant cells were fibroblasts, yet cells of the macrophage type evidencing phagocytosis were observed.

The titer of vaccine virus in the flasks with frozen (F) and unfrozen (N) tissues was as follows: N1 = 1:500, N2 = 0, N3 = 1:5,000; F1 = 0, F2 = 0, F3 = 1:50.

The results of the above experiment indicate that vaccine virus did not multiply in the presence of kidney tissue killed by freezing and thawing, while it did persist or increase in amount in cultures set up with normal kidney tissue in a mixture of serum and Tyrode's solution. Furthermore, it is quite obvious that living cells persisted for at least 3 days in Maitland's medium.

Experiment II

Feb. 13, 1929.—Cultures were prepared with frozen and unfrozen tissue as described in the previous experiment. From the respective mixtures 2 cc. were placed in each of 4 Carrel flasks. The titer of virus at this time: frozen = 1:1,000; normal or unfrozen = 1:1,000.

Feb. 16.—10 bits of tissue were removed respectively from each of 2 Carrel flasks containing unfrozen (N) tissue and from each of 2 flasks with frozen (F) kidney, washed in Ringer's solution, and subcultured in plasma and embryo extract. The results of the subcultures were as follows:

N1: 8 showed growth of cells, while 2 did not.
N2: 5 " " " " " 5 " "
F2: 10 " no growth of cells.
F3: 10 " " " " "

Feb. 18.—To test again the viability of cells, 20 subcultures were made from N3 and N4 respectively and 10 from F1 and F2 respectively. The results of the tests were as follows:

N3: 14 showed growth of cells, while 6 did not.
N4: 15 " " " " " 5 " "
F1: 10 " no growth of cells.
F2: 10 " " " " "

Titration of virus from 2 Carrel flasks containing unfrozen (N) tissue and from 2 containers with frozen (F) kidney resulted as follows: N1 = 1:1,000, N3 = 1:100,000, F3 = 0, F4 = 0.

From the results of Experiment II it is evident that certain cells are able to survive for at least 5 days in a mixture of serum and Tyrode's solution. It also appears that vaccine virus survived or multiplied in the presence of living cells while it ceased to be active in the cultures prepared with tissues killed by freezing and thawing.

Experiment III

Feb. 25, 1929.—The vaccine virus used in this experiment was obtained from N3 of Experiment II. It had been diluted ten times and stored on ice for 7 days. As previously described, cultures were prepared with frozen and unfrozen minced kidney tissue. Prior to distributing the cultures in flasks, the following controls were set up on mica coverslips:

10 cultures of frozen tissue in plasma and embryo extract.
10 " " " " " serum and Tyrode's solution.
10 " " unfrozen tissue in plasma and embryo extract.
10 " " " " " serum and Tyrode's solution.

Subsequent examinations of the above controls revealed the following facts: None of the frozen tissues showed growth; 9 of the unfrozen tissues in plasma and embryo extract presented signs of growth; 5 of the unfrozen tissues in serum and Tyrode's solution showed evidences either of definite cell growth (Fig. 3), of wandering out of surviving round cells, or of beginning giant cell formation (Fig. 4) through cell apposition.

March 2.—30 pieces of unfrozen tissue were taken from each of 2 Carrel flasks (N1 and N3) and 10 of frozen were removed from each of 2 containers (F1 and F2). To test the viability of cells, these bits of tissue were washed in Ringer's solutions and subcultured in plasma and embryo extract. The results of the tests were as follows:

N1: 10 cultures showed growth, while 20 did not.
N3: 29 " " " " 1 " "
F1: 10 " " no growth.
F2: 10 " " " "

At the beginning of the experiment, titration of the virus resulted in no vaccinal reactions in the rabbit. After incubation at 37°C. for 5 days the contents of the flasks were examined for the presence of vaccine virus. None was found.

The virus used in this experiment was culture virus from Experiment II. It had been diluted and stored on ice for 7 days. During that time it had ceased to be active. This experience coincides with the findings of Eagles and McClean (6) who have had difficulty in preserving culture virus. The results of Experiment III indicate that cells are not only able to survive but may at times multiply in a mixturc of serum and Tyrode's solution.

DISCUSSION

The results of the experiments described above are in agreement with those obtained by other workers who found that tissues killed by freezing and thawing failed to support *in vitro* the multiplication of vaccine virus. No evidence was secured to cast doubt upon Maitland's observations concerning the increase of vaccine virus in a medium consisting of minced fresh normal kidney tissue suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts). In this medium, however, which Maitland considered not to be a tissue culture and in which he thought autolysis of the cells to be extensive within 3 days, it was possible to show that many cells remain viable for at least 5 days. This was accomplished by subculturing bits of the

tissue in a favorable medium of plasma and embryo extract. Furthermore, it was found that a medium of serum and Tyrode's solution is capable at times of supporting multiplication of certain cells (Fig. 3).

From the work here presented it appears that the increase of vaccine virus obtained by Maitland did not occur in the absence of living cells. Nevertheless, Maitland has made a definite contribution to the study of viruses in that he has found a medium for the easy cultivation *in vitro* of vaccine virus and other infectious agents of a similar nature (7).

SUMMARY

Cells survive for at least 5 days and at times are capable of multiplying in a mixture of serum and Tyrode's solution used by Maitland for the cultivation *in vitro* of vaccine virus.

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EXPLANATION OF PLATE 7

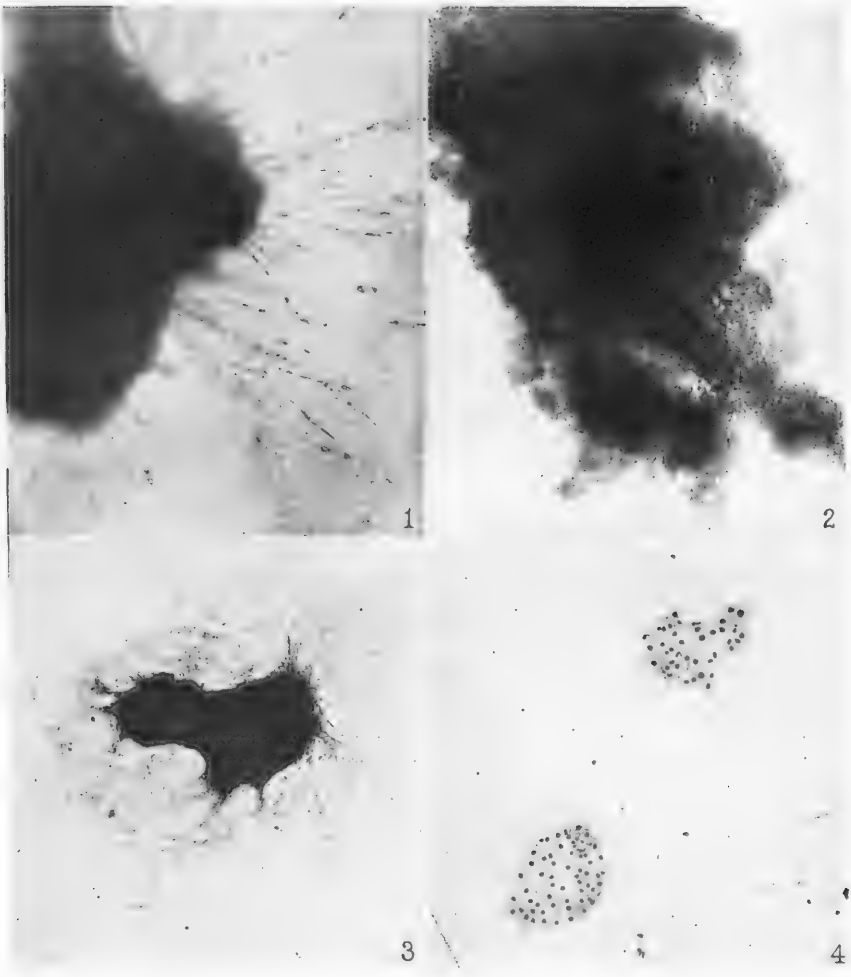
FIG. 1. A small piece of kidney tissue removed from a 3-day Maitland culture and subcultured in plasma and embryo extract. Note growth of cells. × about 95.

FIG. 2. Tissue treated similarly to that in Fig. 1 with the exception that it was

frozen and thawed before being used in a Maitland culture. Note absence of cell growth. × about 95.

FIG. 3. Fresh kidney tissue cultured 4 days in a mixture of serum and Tyrode's solution on a mica coverslip. Note growth of cells. Fixed preparation stained with Delafield's hematoxylin. × about 85.

FIG. 4. Giant cells formed by cell apposition in a medium of serum and Tyrode's solution. Fixed preparation stained with Delafield's hematoxylin. × about 85.



(Rivers *et al.*: Persistence of living cells in Maitland's medium)

STUDIES OF TISSUE MAINTENANCE

I. The Changes with Diminished Blood Bulk

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Plate 8

(Received for publication, June 10, 1929)

The present paper is the first of a series dealing with the service rendered to the tissues by the blood under various conditions. As indices to such service we have utilized the extravascular spread of easily recognizable, innocuous materials thrown into the blood stream. For the purpose of the work here reported certain highly diffusible vital dyes have been employed.

It goes without saying that the multifarious activities of the blood in relation to the tissues cannot be adequately comprehended through observations on the passage from and into the vessels of any single substance or set of substances. But one can at least obtain in this way a knowledge of the general problem in some of its quantitative aspects. Most of the information thus far accumulated concerning it is inferential in nature, being the outcome of observations on rates of blood and lymph flow, on the relative abundance of capillaries in different organs, on capillary pressures, and the state of the local circulation as determined by direct observation. Hooker, Krogh, Richards and others have made studies of the small blood-vessels which illumine the general field; and some investigators have followed directly the diffusion from or into individual capillaries. Our aim has been to determine what the blood does under pathological conditions for the various organs of the body as a whole.

The Choice of Materials

The peculiarities of living cells as manifested in what they reject or accept, and accepting, utilize, secrete, or store, renders the problem of tissue maintenance highly diverse. But it should be possible to

find out in a general way whether the circulation is adequately serving the cells in bringing materials to them and away from them, irrespective of what the cells do with these materials. Susbstances that are let pass by the capillaries must, of course, be employed. True, these will be let pass in varying degrees. But it would seem to be a general law that diffusible, non-toxic "acid" stains penetrate the capillary wall at approximately the same relative rates as when they spread through gelatin (1). If a number that differ widely in this latter respect are selected for animal test it should be possible to gain an insight into how the tissues are served with materials normal to them. At first thought one would say that normal substances should be used. But not only are many of these subjected to change or removal through cell activities, with result that the gradient of permeability is altered locally, but their situation and quantity cannot be gauged with the eye as it can in the case of vital stains of intense color. The best of these stains have special affinities and are stored or excreted in ways that alter interpretations. But with the recognition of such peculiarities, errors due to them can be ruled out. The monographs of Schulemann (1) and of von Möllendorff (2) give one access to a large series of vital dyes. From amongst them and from other sources it should be possible to select test-substances covering the diffusion range of most materials normally purveyed to the cells, with exception of the gases.

The recent history of vital staining is an instructive one, illustrating as it does how rapidly the uses of a scientific tool of wide applicability can become stereotyped as result of success with it in a special field. Ehrlich brought vital staining to modern attention, making significant observations on the nervous system with the aid of methylene blue about thirty years ago (3). But with the subsequent discovery that certain of the poorly diffusible dyes are taken up and stored within living cells, interest turned almost wholly in the direction of the disclosures thus made possible. Not very often since, despite the immense gamut of available dyes, has diffuse vital staining been employed, and then, with a few noteworthy exceptions, for highly specialized purposes. Furthermore the range of dyes utilized in the study of cell-storage has of late narrowed instead of broadening, the mass of investigations nowadays being conducted with one or another of but a few slightly diffusible stains. An observation of the earlier investigators should be mentioned because of its bearing on our own problem, namely that dye storage is especially abundant in the eye muscles, diaphragm, and heart,—whence these workers inferred the existence of an especially great fluid interchange in the organs mentioned (4). Recently Okuneff (5) and Kusnetzowski (6), using the same

criterion, have concluded that much more dye reaches the cells of regions that are inflamed or heated than is the normal case.

For the present work highly diffusible stains devoid of confusing tissue affinities have been selected—Patent Blue V, brom phenol blue, phenol red and sodium indigotate. In enlargement of some of the observations india ink has been pressed into service. Ordinarily we have followed only the distribution of the dyes from blood to tissues, not their subsequent removal from the latter.

Patent Blue V is an intense stain of great diffusibility (7).* Within a few seconds after it has entered the blood stream white animals become brilliantly blue; and by pressing out the blood from the tissues the extravascular situation of the dye can readily be demonstrated. To obtain data on its rate of diffusion into the various organs is difficult, so quickly does it enter most of them. One can always tell, though, where it is and where it is not; and it has the great advantage of rendering visible the walls of the arterioles. Unanesthetized rabbits injected with 1½ cc. per kilo of a warmed, unbuffered 8 per cent solution of Patent Blue V (Hoechst), of pH 6. approximately, manifest no symptoms whatever; and etherized ones show no disturbances of heart beat or blood pressure that are observable with the kymograph, other than those fleeting ones produced by a similar quantity of salt solution. The dye retains its color within the organism. More than three-fourths of it can be recovered as such from the urine of the first twenty-four hours, and most of the rest later on.

Brom phenol blue, somewhat less diffusible and leaving the blood not quite so rapidly, is almost as intense a vital stain. Its range as indicator lies too far to the acid side for the blood and tissue reactions to affect its hue. For our purposes it has proved well-nigh ideal, being devoid, when properly purified†, of action on blood vessels or heart, and diffusing at such rate that the stages in its distribution

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* A correlation of the diffusibilities of the dyes *in vivo* and *in vitro* is being carried out in our laboratory by Dr. Frederick Smith who will report upon them later. It will suffice here to state that a watery solution of Patent Blue V diffuses through a porous glass disc of the sort employed for diffusion measurements by Northrop and Anson (*J. Gen. Physiol.*, 1929, **12**, 541) about as quickly as does dextrose. When the pores of the disc have been filled with gelatin it passes only about one fourth as rapidly as dextrose.

† As ordinarily sold for pH determinations brom phenol blue often contains contaminants affecting the blood pressure. Hynson, Westcott and Dunning have most kindly made for us a purification of their product, which has proved innocuous and devoid of cardiac or vascular effects in cats and rabbits.

can be followed in animals killed at brief intervals after injection.* Furthermore its color so dominates over that intrinsic to the tissues that direct comparisons of degrees of staining become possible. It is best employed in 4 per cent solution, and for intense staining a somewhat greater fluid bulk than of Patent Blue V must be injected intravenously, 3 cc. per kilo of cat or rabbit.† Our usual technic, as in the case of other stains, has been to inject the warmed dye solution during the course of one minute. By the end of this time the body surface is already an even, intense blue; and the failure of the tissues to change color when the blood is driven from them by pressure shows that the dye has already largely passed from the vessels. Neither then nor later are symptoms evident. Rabbits suddenly stained deep blue and placed on the floor sniff about inquiringly and when food is placed before them at once fall to. Three minutes after the dye injection the staining is nearly at a maximum throughout the animal, though accurate color comparisons show that it deepens slightly within another five minutes, remaining constant then for approximately 4 hours and gradually fading later. By next day the animal is practically decolorized, the dye lingering only where the circulation is poor, as in the cartilage of the ear which is still light blue, the tendons (paler blue), the sclerotics (faintly blue); or about a locus of special retention as in the case of the blue gall-bladder wall through which resorption is still taking place secondarily from bile blue-black with the dye excreted with it. At this time the blood plasma no longer is colored. The phthalein leaves the body chiefly by way of the kidney though a little escapes in the feces. As much as 94 per cent of the amount injected has been recovered in the urine of the first 48 hours. The liver takes much out of the blood at an early period, secreting it into the bile, but, as happens with so many other stains, it passes into the circulation again from the gall bladder and intestinal tract, and ultimately escapes in the urine after all. No evidence has been obtained of the least decolorization of the dye within the organism. If any occurs it is negligible.

Phenol red diffuses twice as fast as does brom phenol blue‡ and somewhat more rapidly than Patent Blue V. Much of what has just been said applies to it. Because of the current utilization of it in renal tests it is readily available in pure

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* Dr. Smith has found it to pass through the porous disc of Northrop and Anson at a speed slightly more than half that of Patent Blue V irrespective of whether the disc has been filled with gelatin or not.

† We are indebeted to Dr. MacInnes for freezing point determinations which show that the solution we have employed is isotonic with 0.92 per cent NaCl, and to Dr. Mirsky for observations with the glass electrode which show the pH to be approximately 7.24 at 37°C. The method of preparing the solution has been given in a previous paper.

‡ Unpublished observations of Dr. Smith.

form; and the injection of a freshly made, warmed, isotonic 4 per cent solution of it at pH 7.4, to the amount of 3¾ cc. per kilo, the optimal quantity for staining, causes no symptoms or cardiac or vascular manifestations that a corresponding amount of Ringer's solution would not elicit. It can be used to study blood service to the connective tissue and the other relatively alkaline matrix tissues (cartilage, fascia and tendon(8)) where it appears ruddy, but not for observations on the muscles and viscera, since in them it assumes various shades of orange and yellow that are not readily perceived and evaluated when only a small amount of the dye is present.

Sodium indigotate is poorly soluble at best, only to 2 per cent in water at body temperature; and as much as 7½ cc. per kilo must be injected if the color native to the organs is to be drowned in blue. It is reduced to indigo white in many of the tissues, though it turns blue again on exposure to air; and it is rapidly excreted by liver and kidneys. For all these reasons it has proved unsatisfactory in the study of blood service, although it can be used in confirmation of certain phenomena.

General Procedure

Rabbits and cats have been used for most of the work, and light ether as the anesthetic when one has been necessary. Many of the rabbits have been unanesthetized. To begin with, the normal distribution to the tissues of brom phenol blue was ascertained, and a routine method of examination was worked out. In order that the spread of the dye to the superficies might be studied the fur was in many instances removed from a large part of the trunk and thighs some days prior to the observations, the animal being guarded against chilling thereafter. Barium sulphide proved better for the purpose than shaving. Areas accidentally inflamed were rapidly discriminated by the special intensity of the staining. Ordinarily the animals were fasted 24 hours in order to avoid a digestive hyperemia of the gastro-intestinal tract (Bier); but they were allowed water. Etherized ones were kept on an electrically warmed pad, and were not stretched out but laid on the back or side, without ties. Tracheal cannulation ensured a more even anesthesia. The dye was injected into an ear vein of rabbits and into the basilic vein or internal saphenous of cats, these vessels frequently being cannulated for the purpose. Oiling the body surface brought out brilliantly the surface hues. Since the abrupt introduction of even as little as 1 cc. of fluid into the circulation of a large rabbit brings about compensatory readjustments (Tigerstedt) the dye solution was given gradually in the course of one minute, as already stated. Ordinarily three further minutes were let elapse and then the animal was killed, by cutting both carotids, or,—when no anesthetic had been used,—by decapitation at a blow. The complication of stained blood within the tissues was minimized by the rapid exsanguination. The autopsy was carried out at once and very rapidly, by two workers, with the animal on a slanting board, head down. The organs of special interest were first looked at, the order of

inspection being purposely varied to rule out the possibility of errors due to post-mortem diffusion of the dye. The intensity of the staining as viewed in the gross, was frequently recorded in terms of Ridgway's color standards (9) according to the method used in previous investigations (10). Save in special instances we have not concerned ourselves with the precise location of the dye within the tissues, the main point being that it should have left the circulation, have been served up to the cells, so to speak, irrespective of acceptance or rejection by them.

Not infrequently an amount of blood equivalent to that of dye was removed just prior to injection of the latter. But needless to say this proceeding merely complicated the issue. For not only must some vasoconstriction have been invoked by it, but the salt solution containing the dye must practically at once have been removed from circulation. Whatever the importance of these various factors they did not suffice to bring about differences in the picture. The animals as a group yielded consistent findings, irrespective of the stain employed.

The Staining in Normal Animals

The phenomena observed after the injection of brom phenol blue were essentially the same in rabbits and cats, and were unaffected by etherization. By the time the injection was completed the *hairless tip of the nose*, the *gums*, *conjunctivae* and *fauces* had stained deeply and evenly. The pads of the feet became blue only a little more slowly and less markedly. The *general body surface* took on color progressively and evenly except for intensifications where vessels were abundant, as over the heels and about the mammae. After three minutes the surface hue was brilliant (between "cadet blue" and "diva blue"—Ridgway), the stain lying in the connective tissue. The thin sheet of *voluntary muscle* coming away with the pelt was but palely blue, much paler than the external and internal oblique and the pectoral and leg muscles. These in turn were not nearly so well stained as the *diaphragm*, *intercostals* and *lingual muscles*, the differences being especially plain when muscle layers of the same thickness were compared. To the unaided eye the staining of *subcutaneous tissue* and muscles appeared diffuse, and this still held true when the animals were killed only a few seconds after the injection. For example the tissues of a rabbit killed within 15 seconds after an injection lasting 30 seconds appeared diffusely stained, as did those of a cat killed 15 seconds after an injection which had itself required 30 seconds. But in certain noteworthy instances of animals kept for the routine period of 3 minutes there were indications in the muscles of a latticing or transverse banding with blue, a phenomenon later found to be pronouncedly present when staining was done after the blood bulk had been reduced by bleeding or through the production of anhydremia (11).

The *connective tissue* and *fascia* were everywhere of a medium blue, the *cartilage*, —except for the rib cartilages which fail to stain,—a lighter blue, and so too with the *tendons* and the newer portions of *bone*. Old bone remained uncolored.

Though the supporting framework of the *adipose tissue* was well colored the fat itself did not stain, nor did the white matter of the *central nervous system*, the gray matter being dubiously tinged. The *nerves* to the muscles were beautifully visible in grayish blue to their finest ramifications. The *red bone marrow* appeared deeply colored, but the dye was localized to the blood content. There was intense staining of the media of the larger *arteries* suggesting a special affinity, though the subsequent decolorization took place nearly if not quite as rapidly as elsewhere save in the aorta where it noticeably lagged. The latticing and banding in the muscles mentioned above was not due to this vascular staining. The walls of the *veins* stained poorly. The fibrin of post-mortem blood clots stood out in deep blue. Embolism and thrombosis were sought for but never found.

The tissues thus far described were only moderately blue as compared with most of the abdominal viscera. These were so suffused with the dye as to afford a startling contrast. The *intestines*, large and small, were a deep purplish blue, and so too with the *oesophagus* and *gall-bladder*. The bladder bile of rabbits was already definitely blue after three minutes, but that of cats only later. The peritoneal surface of the *stomach* appeared rather light blue, though finely stippled with darker blue points in the case of the cat; but when the organ had been slit open the mucosa and submucosa proved to be deeply stained, like the gut further down. It was the gastric muscularis into which relatively little of the dye had gone. Occasionally there were to be noted in it areas of local contraction which had not stained at all though the inner and outer layers of these areas had stained as well as ordinary. In this connection the fact deserves mention that the segments of large intestine distended with fecal masses were as excellently stained as the empty, contracted regions between. A notable example was furnished by the rabbit colon wherein fecal pellets are usually distributed at nearly regular intervals with a thinned wall over them and contracted gut between, like coarse beads on a thick, gristly string. Such a colon when slit longitudinally, emptied and inspected between glass plates had the same color intensity everywhere.

So rapidly did stain pass into the *mesenteric lymphatics* that they were distended with deep purply-blue fluid within 15 seconds after the dye injection. The glands at the root of the mesentery contained a similar fluid. In view of the extremely rapid diffusion the viscera were ordinarily inspected first of all, often before the heart had stopped.

In the gross the *liver* appeared deeply and evenly colored, but Valentine knife sections disclosed minor variations in hue, the periphery of the lobules being more intensely colored than the center, and both appearing greenish as compared with the clear blue of the interlobular connective tissue. In rabbits killed after three minutes some stain had already reached the bile. The *spleen* was a more or less deep blue,—less when the organ was somewhat contracted. The *kidney* cortex was dark blue, the medulla lighter, and brom phenol blue was present in the cortical tubules. The *urinary bladder* was medium blue, irrespective of whether it was full or empty, the hue approximating that of the superficial connective tissue. The *omentum* of the cat was lighter. That of the rabbit proved too filmy for useful

observations. Both in omentum and *retroperitoneal* fat the stain was localized to the connective tissue framework. The *pancreas* appeared evenly and rather lightly stained. Here too the color was principally in the connective tissue.

The *ovaries* stained rather intensely, and so too with the medulla of the adrenals, the cortex staining scarcely at all. The *pregnant uterus* was deeply blue, but into amniotic fluid and well-developed fetuses the dye had not penetrated during the few minutes following the injection.

The *lungs* were evenly and lightly blue, and the *thymus* too. The *heart wall* appeared deep purple blue. We have not attempted to determine how much of the color was due to contained blood.

In significant contrast to the mesenteric *lymphatics and glands*, those of the limbs contained fluid that was at most but palely blue.

Substantially identical findings were obtained with Patent Blue V, though no banding or lattice work was disclosed in the muscles, so rapidly did the dye diffuse. With sodium indigotate, the far greater intensity of the staining in the viscera was readily demonstrated, but minor differences were not easily to be apprehended owing to the reduction of the dye to a colorless form.

A number of the organs considered above will not be referred to again in the course of the present paper, notably the heart, kidneys, thymus, ovaries and adrenals. We have described them merely to round out the picture.

In summary one can say that certain organs or tissues (brain, lamellated bone) are entered practically not at all by the dyes of our experiments, that others receive but little of them (cartilage, tendon, new bone, nerves), others show them in considerable quantity (connective tissue, urinary bladder, muscle, pancreas), while others yet become so suffused as to constitute a group apart (liver, gall-bladder, intestines, stomach). The deep color of the spleen and red bone marrow is deceptive, the dyes lying for the most part still in the blood contained in these organs. In not a few others they are localized almost entirely to connective tissue scaffolding and interstitial fluid. With the binocular microscope one can readily make out that the epithelium of the gut is stained only faintly if at all, and that in voluntary muscle the color lies in general between the fibres. But, as has already been stated, our object has been merely to determine whether the dyes are purveyed to the cells, not whether they are taken up. The observations just recounted yielded a norm for the distribution from the circulation.

Certain affinities of brom phenol blue require recognition at this time. It has some affinity for the media of arteries, as also for medul-

lated nerves. In common with many other dyes it is rapidly removed from the blood by liver and kidneys. Möllendorff has correlated the rate of excretion of such dyes into the bile with their physical properties (12); yet the activities of the liver cells in secretion of them are still not wholly understood. The failure of the phthalein to penetrate old lamellated bone is evidently due to the constitution of the latter. The failure of the brain to stain with vital dyes in general has never been satisfactorily explained. The intense color of the diaphragm, intercostals and tongue as compared with the other skeletal muscles is understandable on the basis of their more abundant circulation (13).

The Technique of Depletion

When the blood bulk has been suddenly and considerably reduced, by bleedings or procedures causing anhydremia, profound alterations take place in the service rendered to the various organs by the circulation. Some of the changes have been briefly described in a report on the local, extravascular acidoses arising out of the state of affairs (14). We shall here consider them more fully.

To deplete the cats and rabbits used in the present observations successive bleedings have been employed. Since the circulatory alterations disclosed by the staining method are seen in pronounced form only when the blood bulk is not restored through readjustments within the body, the animals were fasted from 18 to 24 hours prior to experiment in order to lessen the utilization of fluid from the gut, as further, to avoid digestive hyperemia. Usually they had access to water; but under the circumstances they drank little. Those that were etherized and connected with the kymograph by way of the carotid were bled, either from this carotid by puncture of the rubber tubing just above the cannula, or preferably from an axillary artery cannulated for the purpose. Rabbits were bled with the aid of local anesthesia (novocain) by a method which involves a preliminary operation under general anesthesia to bring the carotid to the surface, cannulate it, and place about it an elastic clamp. The clamp was made from a short piece of rubber tubing about 3 mm. in outside diameter which was bent upon itself and tied so tightly at the bend that its limbs did not lie in parallel but sprung somewhat apart. The contrivance was slipped about the artery, and the free ends of the tubing were pulled through a constricting ring of rubber formed from the segment of a larger, thick-walled tube. To shut off the vessel the ring was rolled toward it along the pieces of tubing, the pressure of these latter upon each other sufficing for the purpose. For bleeding the ring was rolled away. There was an optimal position of the latter at which the vessel was held gently shut, to be opened merely by pressing the ends of the contrivance toward each other, thus

springing its sides apart. Successive bleedings were readily carried out with the animal on its feet.

It was early found that a large depletion, especially in the absence of general anesthesia, was required to bring about deviations in blood service so pronounced that certain regions which ordinarily color well failed to stain. No effort was made to determine the least loss of blood that would suffice for this purpose. When it was accomplished by a progressive anhydremia the blood pressure often varied little from the normal (15); but when bleeding was employed it usually fell to about 100 mm. Hg in cats, and 60 mm. Hg in rabbits, in the absence of any efforts to conserve it. After a first hemorrhage, as is well known, fluid from the tissues enters the vessels; but this readjustment is ordinarily completed within a few minutes, and takes place to but a slight extent after later bleedings (16). To rule out its influence upon the findings, as also to permit conditions to become relatively stable, three or four bleedings were done in all, at ten to twenty minute intervals, and the dye injection was ordinarily deferred until twenty minutes after the last one. Great care was taken that the depilated, depleted animals should not grow cold during the experiments. The unanesthetized ones were kept in warm rooms, and those under ether were in addition placed on electrically warmed pads. With the successive bleedings the skin and mucous membranes became pallid, and the superficial veins more or less collapsed. Very occasionally an ill-defined surface mottling could be made out.

Ordinarily almost half, if not quite half, of the calculated blood volume (which is approximately 7.5 per cent in the cat, 5.5 per cent in the rabbit) was removed. The rabbits depleted with local anesthesia still kept their feet but the respirations were exaggerated. That the alterations in the staining were not traceable to low blood pressure, as such, had been disclosed by the observations on anhydremic animals (17), and was now further shown by the fact that the longer the interval elapsing between the final bleeding and the injection the more pronounced were the deviations from the ordinary staining, although in the interval the pressure often tended to recover. The same amount of dye per kilo was given as under normal conditions, and in the same way. It sometimes caused a partial recovery of the blood pressure, but no symptoms. As in the case of the controls, the animals were killed three minutes after the injection.

The Alterations after Reduction of the Blood Bulk

The phenomena occurring in the *superficial tissues*, in the pelt that is to say, of animals receiving brom phenol blue have been briefly described in a preceding paper. The cat or rabbit turned blue more slowly than usual, the rate depending on the degree of depletion and how long after it the dye was injected. In extreme instances only a faint blue staining developed and this was limited to the regions where large vessels entered the skin. In the majority of instances the staining was patchy, unstained areas being everywhere interspersed amidst others that became brightly and diffusely blue. This patching proved in the

cat to be essentially similar to that already described for the rabbit and rat (18) but the white areas tended to be larger. They were irregular in outline, with serpiginous margins, frequently confluent, and varied in size according to the depletion conditions, the arrangement being sometimes of blue on a white ground and again of white on blue. In poorly marked instances there was merely a scattered sprinkling of small white spots on the blue expanse. The patching was especially well seen in well-nourished white cats with an underlying panniculus. In extreme instances, in which phenol red had been used instead of a blue dye, there took place a very gradual orange staining,—the color indicative of acidosis,—about the largest arteries entering the skin, while elsewhere the surface was unstained. The orange-red hue of the conjunctiva in such cases pointed to a blood acidosis. In less severely depleted animals the injection of the phthalein was followed by a brilliant mottling of red on white or white on red. Where the dye crept in later, at the edges of the unstained regions, it was seen to be orange.

Slight differences in local pressure sometimes exerted a great influence to determine the situation of patches in the depleted animal (19). Crouching rabbits often showed a broad, unstained strip along the ridge of the backbone and other large unstained areas over the bulge of the knees. It was necessary to allow for such localizations, as further for the influence of isolated masses of fat to make an overlying, thin, translucent skin appear poorly stained. Where errors of interpretation from these causes could be ruled out one saw that the patches were not only highly irregular in contour but without trace of symmetry. On reflecting the pelt the relation of them to the blood vessels could be made out; and the arrangement of the patching was found to be wholly independent of the vascular pattern visible in the gross, save in the extreme instances above mentioned in which only the tissue immediately about the largest vessels was stained. This independence was especially well to be seen where a number of arteries supplied the subcutaneous tissue in parallel, the lumbar vessels of the cat, for example. Here some of the arterial twigs ran to patches that were white, other corresponding ones to areas that were blue, and yet others to areas that were irregularly blue and white (Fig. 2).

The patching was not essentially dependent upon cooling of the skin, though frequently developing when it was cool. It appeared pronouncedly in one of our animals which was accidentally overheated on the pad; and it can be regularly elicited in rats submerged in oil at body temperature (20). If nothing occurred to relieve the depleted organism the white areas persisted for long periods, though tending gradually to diminish in size by a peripheral encroachment of the dye. When they were very large dye sometimes appeared secondarily at spots here and there within them. Obliteration of them occurred in both ways when the blood volume was restored by reinjection of the portion removed.

The *gums*, *nose tip*, *conjunctivae* and *mucous membranes of the mouth* colored promptly and deeply even when they had appeared absolutely bloodless prior to injection of the dye. They showed no patching, nor did the pads of the feet, which also stained rapidly but somewhat less well.

The *skeletal muscles* exhibited a remarkable reticulation or transverse banding with blue as has already been briefly recorded. And the pattern, unlike that of the skin, was regularly ordered, and did not vary in its dimensions with the degree of depletion. It could be studied directly under the binocular dissecting microscope in certain of the flat muscles (tibialis anticus, gracilis, pectorals, extensor longus digitorum) wherein it took the form of a transverse banding, and in others (as e.g. those of the abdominal wall) where a blue reticulum separated oval, unstained areas. To obtain specimens glass plates were slipped over and under the muscle which had been loosened from the tissues sufficiently for this purpose, and the attachments were then cut. The banding was now seen with the unaided eye to be discontinuous, consisting of many short blue segments displaced regularly a little to this side or that of the main axis of the band. When the muscle was thick (quadratus lumborum) the segments were frequently superimposed at different levels. The blue reticulum was likewise discontinuous. For the present it will suffice to say that the arrangement of the staining was determined by that of the vascular tree, and that it occurred about vessels of a special magnitude, several times larger than capillaries. The average length of the latter in rabbit muscle is 0.69 mm. (21), while the unstained regions between the blue bands (or blue reticulum) were more than 2.0 mm. across in this species, and even broader in cats.

Greater differences were evident in the staining of the various muscles than under normal circumstances. The cutaneous muscle layer did not color at all after severe depletion, and the sheet muscles of the abdomen but slightly as compared with the brilliantly banded quadratus lumborum and gracilis. Even these were pale in comparison with the diaphragm, intercostals and tongue muscles which were diffusely stained and almost if not quite as deep blue as ordinary. Sometimes the intercostals showed a slight relative pallor midway between the ribs; but the blue was uniformly distributed in diaphragm and tongue even when the animal had been sacrificed only a few seconds after injection.

The findings thus far described bear witness to pronounced and peculiar empoverishments of blood service. Not so with those in the *gastro-intestinal tract.* Here the staining appeared to have the same intensity as ordinary, and only on recording the hues in terms of Ridgway's book did one perceive it to be slightly less. As result of the fasting the duodenum and jejunum were wholly collapsed as a rule. The *intestines*, large and small, were always a diffuse, even, purply blue, and so too with the *gall-bladder* wall, *esophagus* and the mucosa of the *stomach.* In extreme instances the muscularis of this last organ showed an ill-defined, slight, pallid blotching but as a rule the coloration was diffuse. The staining was as good where the intestines were stretched over fecal masses as in the contracted lengths between. All this was true even when the animal was killed practically at once after the dye injection. Already the *lymphatics* of the mesentery and of the gall-bladder wall were distended with deep blue lymph.

The *liver* was, as in the normal animal, diffusely and deeply blue. The *pancreas*

was stained about as well as ordinary and showed no patching. The *urinary bladder*, on the other hand, was decidedly paler than in controls, often indeed almost unstained, but without any patching. And the condition of the spleen showed frequently that it had been shut off *in toto* from the circulation, even in etherized animals. Contracted and small the organ stood forth in vivid red against deep blue surroundings. When depletion had not been severe small spreading areas of blue were found scattered amidst the red of the surface exposed on section.

The *omentum* of cats presented a singular picture, being splotched with blue and white wherever it was thick enough for the presence or absence of staining to be made out. The distribution of the stain was irregular, as in the case of the skin, and independent of the vascular patterning visible in the gross. It was confined to the connective tissue. The unstained patches were often several centimeters in greatest diameter. In the retroperitoneal fat a similar splotching or marbling with blue was brilliantly evident. The omentum of the rabbit proved too filmy for satisfactory study.

A contrast to these evidences of ischemia was furnished by the *uterus* of cats far pregnant. The organ was found markedly and evenly stained despite the severest depletion, and the veins coming away from it contained much deeply colored blood although there was little elsewhere owing to the exsanguination at death. In this connection mention may be made of the fact that inflamed areas in the skin of depleted animals stained excellently.

The *kidneys* were diffusely blue, to the naked eye at least. *Tendon* and *cartilage* (ear, knee joint) colored somewhat less well than in controls, but there was no patching. The deep hue of the *red bone marrow* (legs, ribs) on the other hand showed that the dye had reached this tissue abundantly. The *lungs* stained lightly, as in controls, save at their wedge edges which were often wholly uncolored. The tissue at the apices was no paler than that elsewhere.

The lymphatics and glands of axilla and groin, in contrast to those draining the intestines, contained a fluid only faintly tinged with the dye.

These various findings were readily confirmed with Patent Blue V, and some of them with phenol red. The patching of the skin could be demonstrated with sodium indigotate, as also the profound staining of the gastro-intestinal tract, gall-bladder included.

In summary of the observations it can be said that under the circumstances of a markedly reduced blood volume certain of the organs continue to be well and evenly served by the circulation, whereas in others a pronounced patchy ischemia occurs. The conjunctiva, lips, gums, pharynx, oesophagus, stomach, intestines, gall-bladder and liver all become rapidly and deeply stained; and the dye appears in quantity in the lymph from the gut and gall-bladder, even

when the carotid pressure has been greatly lowered by the bleedings. Liver lymph was not studied. The red bone marrow continues to be excellently served by the blood in animals at the extreme of depletion, and so too does the pregnant uterus. The skin on the other hand and the voluntary muscles, with certain notable exceptions, are largely deprived of effective circulation. The deprivation takes a singular form, regions showing it being interspersed amidst others in comparison excellently served by the blood as evidenced by the staining. The size and number of the patches in the skin varies directly with the degree of depletion and the length of time it endures. Their distribution is entirely unsymmetrical and they seem unrelated to the vascular patterning visible to the eye. In the muscles on the other hand there is a regular disposition of unstained and stained regions, the latter situate about vessels of a special, and not inconsiderable magnitude. The unstained regions have a diameter several times greater than the length of the individual capillary. Not all of the muscles suffer this neglect. The diaphragm, intercostals and tongue muscles continue to be excellently supplied by the blood, as the depth and evenness of the staining attest.

Though blood service is remarkably well sustained in most of the abdominal organs there are certain significant exceptions. The spleen is largely, sometimes entirely, deprived of circulation, as shown by the failure of dye to enter it, and the urinary bladder, lightly colored in the controls, is often practically unstained in the bled animals. In the omentum pallid, ischemic regions are scattered irregularly amidst others well supplied from the blood as shown by their brilliant blue color.

Observations with India Ink

It has seemed important to determine the precise relation of the blood vessels to the ischemic patching just described. For this purpose we have resorted to india ink injections. A principal result of the work has been the recognition of how far the method falls short of demonstrating circulatory conditions within the body.

The distribution of india ink by the blood stream has been followed by a host of workers. Krogh has made large use of it in his studies of capillary regulation. Our own observations with ink of the sort he employed (Pelikan Perl Tusch,

Günther Wagner), dialyzed against Ringer's solution and filtered in the way he describes (22), would seem to indicate that his findings were obtained in the face of serious technical draw-backs. When the amount of ink that Krogh employed is injected intravenously into a rabbit or a guinea-pig, and the vessels of the ear are watched under a microscope, one can perceive that the foreign particles tend to agglomerate into lumps as they are carried along. These lumps soon lodge here and there within or at the entrance to capillaries, effectually blocking them. Using such material one can be certain only that where it passes the vessels are open. There is, fortunately, no difficulty in demonstrating with it that many more such vessels are open in the diaphragm of the normal animal than in most of the other skeletal muscles, a phenomenon emphasized by Krogh. But to assure oneself that where the ink does not penetrate no circulation had existed is quite another matter. And this holds true even when an ink far better for the purpose is used, Higgins' American Drawing Ink (non-waterproof), dialyzed against Ringer's solution, filtered and centrifuged—the last two processes being unnecessary in our experience. On injection of this the particles can be seen to circulate separately during the brief period before they are removed from the blood by the sessile phagocytes.

Normal white animals receiving either Higgins' ink, or Pelikan Perl Tusch, become transiently gray. We had supposed that in depleted ones patchily but intensely colored with brom phenol blue, injected ink particles would pass into the stained regions of skin and muscle in sufficient quantity for histological recognition if the animals were killed while they still circulated. But this did not prove to be the case. To all intents and purposes the particulate matter was shut off from the organs mentioned, and this proved to be the case as well in animals that had been merely depleted, not stained. The skin did not turn gray anywhere. The abdominal viscera on the other hand were dark with ink, except for the spleen which contained practically none. The liver was black with that which had been taken up by the Kupffer cells. In contrast to the pallid state of the skin generally, the tip of the nose, and the pads were gray with ink, lying doubtless within the arterio-venous anastomoses there known to exist (23, 24). The quantity injected was only $2\frac{1}{2}$ cc. per kilo, because of the need to avoid large increases of the blood bulk. Further findings will be detailed in a succeeding paper.

DISCUSSION

The method of the present work would seem to be validated by the disclosures it has yielded. The alterations we have observed in the

service rendered by the blood to the skin, muscles, and certain other organs of the depleted animal cannot be apprehended by ordinary laboratory procedures; nor are they demonstrable with india ink. In animals depleted by large hemorrhages a rapid and deep staining occurs in some regions (lips, gums, tip of the nose) which under such conditions in unstained animals appear wholly bloodless; and furthermore staining occurs in skin regions to which india ink particles are not carried by the blood. It seems probable that in some of these situations only a stained plasma may have circulated, red cells being removed by the "skimming" that Krogh first described. The great diffusibility of the dyes we employed proved in some ways a disadvantage; for they were so readily distributed that only drastic reductions in service to the tissues were recognizable with their aid. To appreciate less considerable changes it will be necessary to employ vital stains that do not leave the blood so rapidly.

Can it be said that where brom phenol blue failed to go all interchange between the blood and tissues had ceased? Scarcely. For Patent Blue V gradually penetrated where brom phenol blue did not. And carbon dioxide, which passes through the tissues with unexampled ease (25), reaches situations inaccessible to other substances. To all intents and purposes nevertheless the circulation had ceased to be effective in the skin areas which brom phenol blue or phenol red failed to penetrate. For an acidosis developed in such areas, one referable to the local accumulation of acid metabolites (26).

In appraising our results a first question is, how far they were conditioned by special affinities of the dyes employed? By employing several of highly different constitution we have tried to minimize this factor; but in another and better way it has been proven unimportant, namely by following the process of decolorization. Those organs which stain most rapidly and deeply with brom phenol blue are, with exception of the liver and kidneys,—which actively excrete the dye,—precisely those which lose color soonest, as could scarcely happen were it fixed upon them as result of a special affinity. The gastro-intestinal tract and the diaphragm become colorless long before the skin does. Brom phenol blue stains the media of arteries with a special intensity; but even here decolorization does not lag notably.

A principal alteration in blood service after hemorrhage is a peripheral vascular shut-down, the blood supply to the viscera being maintained at the expense of that to the superficial tissues and the muscles. This readjustment has long been recognized as one of the means whereby the vital forces are conserved in individuals "bled white." Other changes disclosed by the staining method impress one with their purposefulness. Not all of the abdominal organs continue to be well served by the blood. Those which are essential, and which can be safely neglected,—the omentum, urinary bladder and spleen,—are neglected. By contrast the whole gastrointestinal tract —from which alone help can come to the organism under natural conditions,—continues to be well served. Special mention may be made of the maintenance of the circulation to the gall-bladder, since the realization is recent that a very active resorption takes place through the walls of the organ into the blood and lymph. Even the esophagus and fauces are well served, and the gums and lips are much better maintained than is the skin. The red bone marrow continues to be excellently supplied with blood, though situated within limbs that are for the rest largely deprived of it.

The amount of blood which would be conserved to the organism were there complete ischemia of the skin is but slight (2 to 3 per cent) (27), but the saving of heat is far more considerable. And the cooling of the neglected tissues lessens the formation of waste products within them. In the voluntary muscles, large, enduringly bloodless patches do not develop as in the skin, but there is a regular arrangement of smaller ones into which a certain amount of diffusion gradually occurs, as shown by the findings with Patent Blue V. But not all of the muscles suffer in this way. Those which are essential to respiration (diaphragm, intercostals) and to swallowing (tongue muscles) continue to be well served by the blood. The question whether this is true because these muscles go on working need not be taken up at the moment, though the fact may be mentioned that the muscles of the tongue continue to be well served from the blood, as shown by a diffuse, deep staining, even when the organ lies flaccid in animals anesthetized through a tracheal cannula.

Under normal circumstances much more stain has been found to pass from the blood into the tissues of the gastro-intestinal tract than into

the skin and muscles. Numerous reasons for this can be thought of. The amount of blood passing in a unit of time through the skin and resting muscles is many times less than through the portal circulation. Capillaries are far more numerous in the viscera while furthermore a great proportion of those existing in the skin and resting muscles are ordinarily shut. There is an active flow of lymph from the blood into the mesenteric lymphatics, but practically none into the lymphatics of resting muscles (28, 29), whence it follows that a dye circulating in the blood penetrates into the muscles only by diffusion, whereas the process of distribution to the tissues of the gut is actively aided by a streaming of fluid out of the vessels. Furthermore the distribution to the gut is aided by a high capillary blood pressure, and the barrier offered by the capillary wall itself is imperfect, as shown by the presence of blood proteins in lymph collected from the mesenteric channels (30). Considering all this one cannot wonder that the stomach and intestines showed a specially intense and rapid staining with vital dyes.

From what is known of the physiological readjustments which take place in the bled animal one might expect the staining in the gastro-intestinal tract to disclose wide deviations from the normal. The capillaries of the region are known to be actively contractile; and a narrowing of the portal channels through vaso-constriction is deemed one of the most important compensatory changes occurring when the blood volume is diminished (31, 32). Even losses of blood which do not suffice to lower the arterial pressure cause some blanching of the intestines (Starling); and when such a lowering has taken place the rate of formation of lymph in the gut and liver is markedly reduced (33). Not a few functional conditions have been described in which blanching of the intestines was so great that the tissue seemed to all intents and purposes bloodless. For every *a priori* reason, then, except the teleologic, one might suppose that blood service to the gut after hemorrhage would be greatly lessened, perhaps to the extent of ceasing in some regions. But the dye experiments showed quite another state of affairs to prevail. Staining of the gut, gall-bladder and liver was always deep, though the animal was at the extreme of depletion and was killed but a few seconds after the dye injection; and the staining was diffuse save occasionally in the muscularis of the

stomach where an ill-defined blotching could be made out. The mesenteric lymphatics were always distended with deep blue fluid. The pancreas likewise was colored as usual. One must conclude that the compensatory constriction was never so great as to interfere seriously with blood service to the digestive organs.

Mention has been made of the fact that the staining of the intestine where it was stretched over fecal masses was of precisely the same intensity as in the empty, contracted segments lying between. The pressure condition where feces distend the gut is probably much like that in the full bladder, of which Sherrington remarks (34) that it "enfolds its contents in the same light grip whether these contents be ample or little." Owings, McIntosh, Stone and Weinberg (35) have ascertained that in normal dogs the greatest intraintestinal pressure is equivalent to only 2–4 cm. of water.

The amount of dye injected into the depleted animals was the same as in normal ones. Since the blood volume had been reduced by nearly half in most instances, it follows that the dye circulated in unusually great concentration. The intensity with which the abdominal viscera stained despite the untoward conditions, must be attributed in considerable part to this cause. But it will not explain the patching of the omentum, the more or less complete failure of the urinary bladder to stain, or the neglected state of the skin and muscles, which, so far as they received blood at all, received the same sort as did the viscera.

Starling has pointed out that normally absorption goes on from the digestive tract irrespective of whether there is a body need for the materials absorbed, the sole recourse of the organism being a regulation through the excretory organs which remove at an appropriate pace that which has been taken into the body willy-nilly (36). It is plain from our findings that even when the blood bulk has been diminished to the limit of tolerance, adequate circulatory conditions are maintained for absorption from the gut, a process which frequently acts to sustain life. Robertson and Bock have proved that salt solution introduced into the intestine is far more effective in permanently restoring the blood pressure of human beings after hemorrhage than when it is thrown directly into the circulation or injected into the tissues (37). The reason for this is not yet clear.

The dyes we have used are rapidly excreted into the bile; and this

of course makes for a deep staining of the liver. Even within so brief a period as three minutes after the injection of brom phenol blue or Patent Blue V, much dye had reached the finer bile ducts. The dyes yielded no evidence that after hemorrhage some hepatic regions were better off than others. Yet the vaso-motor regulation within the liver is far from negligible (38); and a "stroking reaction" can be elicited on the surface of the organ, like the cutaneous one so much studied (39). In view of all this one of us has made a special study of the character of the hepatic blood service after hemorrhage. The results are detailed in the paper which follows.

The mackerel-sky or lattice work staining in the muscles of bled animals was obviously related to the arrangement of the vascular tree. That it was largely dependent upon contraction of the vessels, was shown by experiments in which vaso-constriction was prevented from occurring in the muscles of a leg by cutting the nerves to it just prior to injection of the dye. In the muscles of a limb so treated staining took place diffusely whereas in those of the control leg the usual mackerel-sky patterning was found. Our many experiments of the sort will be described in detail on another occasion. Not infrequently normal, stained cats and rabbits exhibited traces of the patterning here or there, more especially in the gracilis and quadratus lumborum. It may with good reason be attributed to that partial vaso-constriction on which maintenance of the normal blood pressure depends, but there are other important conditioning factors as will be shown subsequently. In not a few cases, just before the introduction of the dye an equivalent amount of blood was removed from the circulation. There was no more pronounced patterning in such instances.

The factors responsible for the patching of the skin are less readily to be explained. They too are dealt with in a subsequent paper. The possibility has already been ruled out (40) that the patching depends on a differing intrinsic permeability of vessels of like magnitude supplying tissue of the same general sort, a difference becoming effective only when the blood flow has been cut down by vaso-constriction.

Some foreshadowings of the changes we have observed in the service to the tissues after depletion can be found in previous work. Meek

and Eyster, watching directly the circulation in the dog's ear, noted that after a considerable loss of blood there suddenly occurs an active contraction of the capillaries and small venules (41). They suggest that possibly "when the circulation is at the breaking point as it is when the bleeding equals 2 per cent of the body weight the venules and capillaries are constricted in widespread areas." The pronounced restriction of blood service occurring in the skin and muscles under such conditions is not uniform, as our experiments show, some regions being still fairly served while neighboring ones are wholly passed by. Langley (42) noted that the circulation continues through a few arterioles in the muscles of the frog after hemorrhages severe enough to stop it in the generality. He believed that a similar state of affairs would be found to exist in mammals. Gesell and Moyle (43), who ascertained the volume flow through the muscles of dogs repeatedly bled, found that at late stages of the gradual depletion it was reduced to an extent out of all proportion to the drop in blood pressure.

The vascular shut down in the spleen after hemorrhage is no new phenomenon (44). One may contrast therewith the state of affairs in the red bone marrow as disclosed by our experiments. Not only does dye still reach this tissue in quantity but india ink does as well.

The conditions as concerns blood service to the kidney under pathological conditions are complicated and we have made no attempts to study the organ. Richards states that relatively few kidney glomeruli are open to the circulation in frogs that have lost blood but that the number can be greatly increased by restoring the blood bulk (45).

SUMMARY

The spread through the living animal of various highly diffusible dyes has been utilized as an indicator of the ability of the circulation to serve the tissues under various conditions. The method is direct and searching. Blood service to the viscera, as demonstrated by it, is normally far more profuse than to the skin and muscles, for evident physiological reasons. After hemorrhages which greatly reduce the blood bulk service to the viscera is in general still well maintained even though the animal be *in extremis*. However great the compensatory contraction of the splanchnic vessels may be,—and physiologists have long supposed it to be very great,—it certainly does not suffice to

hinder blood service anywhere in the digestive tract. On the other hand the service to certain unessential abdominal organs (spleen, omentum, urinary bladder) is cut off in large part or wholly; and in comparison with the essential viscera, the skin and most of the skeletal muscles of the bled animal are largely deprived of circulation. This neglect takes a curious form, some regions being still fairly served by the blood while others next them are no longer ministered to. In the skin the areas served, or not served, are highly irregular but are to some extent determined in situation by local pressure factors. Within the muscles the neglect is orderly in arrangement and is largely referable to compensatory vaso-constriction. Certain of the muscles, those used in respiration and in swallowing, furnish significant exceptions to the general rule, being excellently served despite the serious general state. The red bone marrow of the depleted organism continues to be well served by the blood even though situated in limbs that are, for the rest, almost devoid of a circulation. The pregnant uterus also is excellently maintained despite the serious general state.

The changes are such as would tend to conserve the forces of the depleted organism and to contribute to its recovery.

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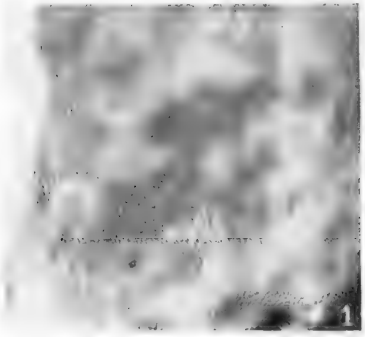
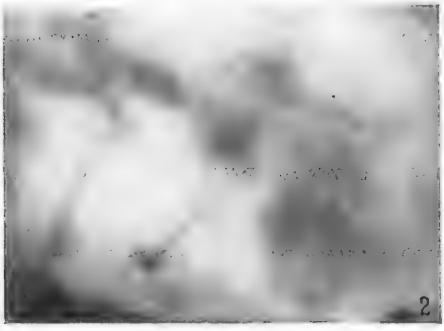
EXPLANATION OF PLATE 8

Fig. 1. Skin of the side of a white cat injected with brom phenol blue after reduction of the blood volume by repeated bleedings under ether. The brilliant blue and white mottling is only moderately well shown in the photograph; yet the contrast is sufficiently great to suggest that the white patches were raised above the blue, as was not the real case. The hair had been removed by shaving.

Fig. 2. Reflected skin of the same cat showing three parallel distributions to the subcutaneous tissue from the series of lumbar vessels. Some of the blue patches in this tissue occupy the regions supplied by one or another of the secondary arterial branchings; but of others this is not true. Three branches that correspond in situation are indicated by arrows. Two of them run to colorless patches whereas the third enters tissue that is heavily stained.





(Rous and Gilding: Studies of tissue maintenance. I)

STUDIES OF TISSUE MAINTENANCE

II. The Service to the Liver and Digestive Tract after Hemorrhage

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Plate 9

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The service rendered to the various organs by the blood can be gauged by the distribution to them of diffusible vital stains. In a previous study by the method, there have been observed certain well-marked alterations occurring after the blood bulk has been reduced by hemorrhage. The service to the organs immediately essential to life and to recovery (heart, lungs, respiratory muscles, alimentary canal, liver, red bone marrow) was maintained at the expense of that to certain others (skin, voluntary muscles in general, spleen, urinary bladder). But these latter organs,—with the exception of the urinary bladder and sometimes of the spleen,—did not suffer to an equal degree throughout, regions in which the circulation was ineffective being interspersed amidst others still served by the blood, as shown by the stain that entered the tissues. It has seemed possible that a similar, if less pronounced, patchy neglect might after all have been present in the splanchnic viscera that appeared well and uniformly served, a neglect masked by rapid diffusion of the dyes, or by a rapidly intermitting circulation, now to this region and again to that. The present work was undertaken to cover the point thus brought up.

One does not have to look far in the literature to find that the vessels of the gut and liver are sufficiently contractile to render the assumption reasonable that a functional ischemia may exist in these tissues on occasion.

Mall showed in 1892 that during stimulation of the splanchnics the blood flow from the systemic arteries to the portal vein is greatly cut down. There occurs

also an active contraction of the portal twigs within the liver (1). These observations on vasomotor activities within the hepatic tissue have been repeatedly confirmed (2). In anaphylaxis of the dog an interference with the circulation through a spasm of the small hepatic vessels leads to the characteristic drop in blood pressure (3). Mautner and Pick (4) have adduced experiments to show that the liver of cats possesses a mechanism whereby contractile spasm of the liver capillaries is produced. According to these authors the action of epinephrin or barium chloride upon the vessels causes obstruction to the blood flow through the cat's liver as not through that of rabbits. Ebbecke brought about a capillary dilatation with edema on stroking the hepatic surface, which he deemed comparable with the cutaneous stroking phenomenon that has of late been so much studied (5).

The fact is well attested that contraction of the portal bed through vaso-constriction is largely responsible for the maintenance of blood pressure after hemorrhage. Krogh has advanced evidence on this point (6). Starling states that a loss of blood too slight to reduce the general blood pressure will cause blanching of the intestines (7). In experiments upon some lower forms an extreme blanching has been elicited in various ways. Bayliss states, in summary of general knowledge, that the innervation of the abdominal viscera is predominantly vaso-constrictor whereas that of the peripheral tissues is vaso-dilator (8). And yet, if one may judge by observations from our laboratory, the vascular readjustment which takes place after large hemorrhages in cats and rabbits, cuts down and may almost abolish the effective circulation to the periphery while interfering but little with that to the viscera.

Method

For the present work india ink was mainly employed. It was mixed with a 4 per cent solution of brom phenol blue or preceded by an injection of the stain, or of phenol red, when information was desired of the state of blood service to the skin and muscles. Though this is brilliantly disclosed with the dyes it cannot be with ink, which, in animals that have been bled, frequently fails entirely to reach the tissues mentioned. Higgins' American Drawing Ink (non-waterproof) was employed. For injections during life this is far superior to the Pelikan Tinte (Günther Wagner) so much used by Krogh, since the particles circulate separately, not in agglomerates of various size. The ink was dialyzed against Ringer's solution for some days and was then both filtered and centrifugalized, processes which separate out few if any particles. 2½ cc. per kilo was introduced at body temperature into a vein, during the course of one minute. There followed only the rise in blood pressure which an equivalent amount of salt solution would have produced. The animals employed were rats, rabbits and cats. In some of the early work general anesthesia was induced with urethane, but this proved unsatisfactory for reasons to be given later. Light ether was employed with all of the cats and they were bled from a cannulated axillary vein. Rabbits can be

repeatedly bled from the carotid under a local anesthetic (9), but most of those employed were etherized. Kymograph records of the blood pressure were taken in many cases. The rats were bled from the carotid while under the influence of urethane or ether.

To learn the distribution of the ink in the stomach and intestines direct inspection during life was necessary; for needless to say much of it might be forced here and there through post-mortem contraction of the vessels. So rapidly are the ink particles removed from circulation by phagocytes sessile within various organs that the inspection has to be carried out within the first minutes after the injection. For these reasons the animals used for the study of the gut were under general anesthesia; and in order to rule out the effects of exposure, the abdomen was opened only when the time had come to look at the organs. In the case of the liver the state of affairs could be studied at leisure after the animal had been killed,—by exsanguination from the carotids, or by decapitation in the case of unanesthetized rabbits; for the ink is so rapidly taken out of the blood by the Kupffer cells that the amount of phagocytosis will serve as an index to blood service. But in order to rule out a possible post-mortem distribution of the ink, with such phagocytosis as result, it was essential to remove the liver from the body immediately upon death. Livers thus treated empty themselves of blood as they do not when they have been left in situ for even a few minutes with the large vessels uncut (10). In the present work the capillaries regularly proved empty. Microscopic preparations were obtained with the Valentine knife or the freezing microtome, and cleared in glycerine. If there was brom phenol blue in the organ, the complication of its intense blue hue was done away with by placing the sections in a solution possessing that slight degree of acidity necessary to turn the phthalein yellow.

The Maintenance of the Liver

In a series of preliminary experiments urethanized rats were bled repeatedly from a carotid; injected with ink; killed after various brief periods of time; and compared with controls merely receiving ink. It was a surprise to find that under these circumstances the spleens of the depleted rats contained as much ink as those of the controls; for Barcroft has proved that the spleen contracts after hemorrhage. Our finding was traced to the urethane, which acts to prevent contraction of the spleen, as Henning showed (11). When the rats had been bled under a local anesthetic, almost no ink reached the spleen. It was everywhere equally distributed to the liver lobuli, irrespective of the character of the anesthesia; but in the bled animals the peripheral Kupffer cells contained vastly more than did the central ones,—which was not the case in controls. In rabbits that were depleted under light ether a pronounced splenic contraction occurred, as proved both

by the appearance of the organ and the almost complete failure of ink to enter it. The rabbit liver on the other hand showed an abundant and even distribution of ink to all of the lobuli, but within these units the same pronounced divergence from the normal was observed as in rats. The peripheral Kupffer cells were swollen with phagocyted ink particles, while those further in had less and less the nearer they were to the center. Identical findings were obtained when the bleedings had been carried out with the help of local anesthesia.

In all of the foregoing instances depletion was purposely carried far, about half of the blood being taken as a rule. Sometimes 20 minutes had elapsed between the last bleeding and the ink injection, sometimes a less period, the plan being not to exhaust the activity of the vaso-motor center. The unanesthetized rabbits maintained the ordinary crouching posture. In every instance in which brom phenol blue had been mixed with the ink a pronounced patchy ischemia of the peripheral tissues was disclosed.

Identical findings were obtained in cats bled and injected under light ether, save for the fact that the ink was almost evenly distributed to the phagocytes within the hepatic lobuli, just as in the controls. Only when the depleted cats were sacrificed within a few seconds after the ink injection, with result that but little had been phagocyted, was more visible in the peripheral Kupffer cells.

The Service Rendered by the Hepatic Artery

The existence of two sources of hepatic blood renders uncertain the interpretation of the foregoing observations. That the two vascular trees supplying the hepatic tissue differ in their vaso-motor responses is known (12); and it might well be that the effects of a contraction of portal radicles here and there would be masked by a freer flow from the end arteries, or vice versa. For this reason observations were made on the distribution of ink after diversion of the portal stream from a part or all of the liver.

In some etherized cats and rabbits the branch of the portal vein running to the "main liver"—the mass lying between stomach and diaphragm—was tied, the result being that all of the portal blood was diverted through the "lobe mass"—the right posterior and caudate lobes. This abrupt change in the path of the blood is well tolerated by the organism, as an abundant experience has shown (13); and

in the course of the present work it has been observed to have no effect on the carotid pressure. Needless to say the necessary operation was performed with the least possible trauma and exposure of the viscera, only a short segment of the vein being dissected free for ligation.* The hepatic artery, in special, was not touched; and it was seen by direct inspection to continue to beat strongly. The abdomen was then closed with a running suture in two layers. After 2 to 18 minutes had been permitted to elapse, for purposes of readjustment within the organism, the bleedings were carried out as usual, and ink injected. When the animal was killed a little later the lobe mass was found coal black with phagocyted ink particles whereas the main liver was of a light or dark brown. In both portions of the organ the ink had been evenly distributed to the lobuli. To control the effectiveness of the ligation milk was injected under pressure into the portal vein below the tie. It flowed only into the lobe mass.

One of the animals, a rabbit, had been vitally stained with phenol red just prior to the portal diversion and its body surface had colored a uniform red. After the bleedings, at the time of the ink injection, the surface had become pronouncedly patched with buff upon red, plain evidence that there existed in the superficial tissues local areas of outlying acidosis, such as result from an ineffective circulation.

In some additional experiments (on rabbits) a preliminary operation was performed to induce a development of portal collaterals by partial obstruction of the flow to the liver, after Drury's modification of Moskowitz' method (14). Some weeks later when the collaterals were well developed the portal vein was completely tied off under ether. There resulted no alteration in the carotid blood pressure. After a brief interval to allow for readjustments the animal was depleted by successive bleedings according to the usual method, and ink, or ink and brom phenol blue, was injected. On sacrifice it was found that the effective blood pressure within the liver had been so greatly lowered by depleting the blood bulk that the slight pressure exerted on the organ by the stomach or ribs of the prone animal had sufficed to prevent the ink from entering certain regions. Elsewhere, though, it had been distributed evenly to the lobuli by the hepatic artery. The milk test showed that the portal stream had been wholly diverted from the liver.

In a final experiment the portal vein in a normal male cat weighing 3025 gm. was tied off, with result, of course, that the animal gradually bled into its own splanchnic vessels, the blood accumulating in the portal regions back of the ligature. The abdomen was closed as usual. The carotid blood pressure sank progressively and within 8 minutes the mucous membranes of the mouth, previously pink, had become pallid. After 18 minutes the animal was bled 20 cc. from the carotid cannula to hasten the depletion. After 35 minutes the carotid blood pressure was very low, having fallen from 150 mm. Hg prior to ligature to 40 mm. Hg. Now

* The separation from its sheath of the portal vein of the cat or rabbit is a nice procedure, best accomplished after longitudinal incision of the sheath, by pressure with a pledget of cotton or gauze held in the grip of a mosquito forceps.

8 cc. of ink was injected intravenously during 35 seconds. The skin failed to color with it at all, but the mucous membranes of the mouth became gray in patches. 19 minutes later both carotids were cut. But little blood came away, nearly all having collected in the portal viscera, behind the ligature. The liver was slaty black with ink which had been evenly distributed to the lobuli by way of the hepatic artery. There was slightly more in the peripheral Kupffer cells than near the center. The milk test showed that the ligature had been occluding.

These experiments demonstrate that arterial blood is uniformly distributed within the liver even when the organism is at the extreme of depletion.

The Service Rendered by the Portal Vein

The literature on contraction of the portal radicles within the liver has already been reviewed. There is much to indicate that it exerts an important regulatory influence on the blood supply to the heart, notably after hemorrhage. The following tests were designed to show whether under such circumstances some parts of the hepatic tissue are served by the blood and others neglected.

In etherized cats and rabbits the hepatic artery was dissected free and ligated, at a single point in the earlier experiments, in the later at two points, above and below the origin of the A. gastro-duodenalis, in order to prevent all flow through collaterals. Two ligatures were placed at each situation, the abdomen was closed as usual, and bleedings and injection were carried out in the ordinary way. It was found, after the bleedings, that the intrahepatic blood pressure had been so greatly lowered that the slight pressure of the ribs sufficed to prevent the blood-laden ink from entering the tissue lying next them. Elsewhere, though, it was regularly distributed to the lobuli. The milk test showed that the ligatures had wholly cut off the arterial flow.

Again no evidence was encountered of a patchy maintenance of the hepatic tissue in animals with a reduced blood bulk. Some little arterial blood may have reached the liver by way of the diaphragm. The supply of arterial blood from this source is so small in the rabbit that necrosis of the liver and death regularly follow ligation of the hepatic artery. In the dog the supply is sufficient to avert this catastrophe.

The Service Rendered to the Intestines

When white animals (rats, rabbits and cats) are injected with ink after marked reduction of the blood bulk the body surface does not

become ashy gray as in normal controls. So few ink particles reach the skin and muscles that the regions of total ischemia in these tissues cannot be discriminated with their aid. As the foregoing experiments on the liver have sufficiently shown, they circulate in abundance through the portal bed. Special care had been taken in these experiments not to expose the intestines, in order to avoid vaso-dilatation due to trauma. The short incision necessary to tie one or the other of the hepatic vessels had been made high up to the left of the mid-line where only the upper surface of the stomach and the under surface of the liver came into view. Nevertheless a dilation of the capillaries of the gastro-intestinal tract sufficient to let ink through into the portal vein might have been an indirect result of the operative procedure. To learn whether in the absence of any such stimulus these vessels let ink through, as further to find whether they are patent everywhere, some animals were not laparotomized until after ink had been placed in circulation. The examination was made during the brief interval before the particles had been taken out of the blood; and heed was given only to the condition when the gut was first exposed. The animals had been fasted to rule out all possibility of the digestive hyperemia described by Bier. The stomach and small intestines of the cats were empty. In rabbits the stomach and large bowel were still distended with roughage after several days fasting.

Always it was found that despite the preliminary depletion the intestines and stomach were uniformly gray with circulating ink; and the blood of the portal trunk black. Such vascular contraction as may have taken place in readjustment for the blood loss had nowhere closed off the capillaries to such extent that ink particles could not course through them; and there was no sign of a patchy distribution of the material. The findings confirmed those with diffusible dyes (15). It can be concluded that such compensatory contraction of the small vessels of the alimentary canal as may occur after hemorrhage does not anywhere even nearly shut these vessels, and that the service rendered by the blood to the tissues is far less affected as a whole than is that to the skin and muscles.

Contractility of the Vessels of the Alimentary Tract

That the failure of the small vessels of the alimentary tract to undergo an occlusive contraction after hemorrhage is not due to any

inherent inability to contract has been shown by tests with epinephrin and pituitrin. After one of these has been given intravenously the blood supply to certain portions of the bowel may be so completely cut off that highly diffusible dyes fail to stain them. Service to the liver, though, as judged by this criterion, is still well and evenly maintained.

A sufficient amount of 1–1000 solution of suprarenin (Metz) (0.05 cc. per kilo, diluted with an equal part of 0.9 per cent saline) was injected into a vein of rabbits or cats to cause a great rise in the carotid blood pressure, and while this rise still endured brom phenol blue in the quantity usual for vital staining (16) was run into the circulation. As a rule a little of the suprarenin solution was mixed with it, the object being to sustain the high blood pressure. To avoid the possibility of cardiac default under the conditions the dye was injected more slowly than usual, in the course sometimes of as much as 2½ minutes. In rabbits the blood pressure rise was succeeded by an irregular fall toward the previous level throughout the subsequent period of 3 to 4 minutes before the animal was killed. In cats on the other hand vagal inhibition of the heart beat caused the pressure to drop before it had risen very far, and frequently brought it below the previous normal. This, of course, did not mean that vaso-constriction had been relaxed. At the time when the carotids of the anesthetized rabbits were cut they were hugely distended, their diameter being often twice the normal,—clear evidence of a peripheral obstruction to blood flow.

In both the species used the service rendered by the blood to the skin and muscles was greatly interfered with by the action of epinephrin, as shown by the slow, slight, and irregular staining. The small intestine, colon, and rectum on the other hand stained as well as normally, becoming deep blue within so short a time as a minute after the dye injection. The liver and gall bladder were also rendered diffusely blue and the phthalein promptly appeared in the bile. On the other hand the mucosa of the stomach of rabbits showed large, pallid, serpiginous areas on a blue ground. These contained neither dye nor blood. They had no obvious relation to the vascular arrangement, and the overlying muscularis was as well stained as ordinary. The caecum showed irregular unstained patches involving the whole thickness of the wall. These likewise were unrelated to the patterning of the vessels. In cats such evidence of visceral ischemia was wholly lacking, although in skin, muscles, and omentum it was pronounced. The cat spleens were not contracted whereas those of the rabbits were.

Very singular was the condition of the mesenteric lymphatics of the rabbits. Their contents became colored with blue as rapidly and deeply as ordinary; but after this had occurred the epinephrin must have induced an irregular spasm of the lymph channels themselves, the consequence being that they had the appearance of broken segments of blue thread, or,—in cases in which they were larger, with bulgings between the valves,—of irregularly contracted blue tapeworms. So great had the spasm been in two animals that the channels had ruptured in numerous places near the gut and there were spreading extravasations of blue lymph. Falta and Priestley (17) have made injections comparable to mine into dogs and have studied the vascular state by direct inspection of the organs. They found the intestines as a whole to be greatly blanched, and inferred that the circulation is diverted elsewhere. In my animals only the stomach and large intestine manifested any ischemia and in them its distribution was patchy. The interference with service to the gastric mucosa may have been attributable, in part at least, to contraction of the muscularis mucosae. Adrenalin stimulates this layer as it does not the muscularis proper (18). The ischemia involving the entire wall of the caecum can only be explained on the basis of vascular contraction.

Pituitrin yielded far more pronounced findings.

Two preparations were employed, Pituitrin (Obstetrical), Park, Davis and Co. and Infundin (Burroughs Welcome and Co.). As in the case of epinephrin, a preliminary intravenous injection was made to bring about vaso-constriction, and when a pronounced rise of the carotid blood pressure showed that this had taken place in the etherized animals brom phenol blue or Patent Blue V was run into the blood stream,—very slowly, else the heart failed and death occurred. For the pituitrin had caused an unexampled rise in the general blood pressure. At the time when the carotids were cut in order to kill the animal they were found distended to double the ordinary diameter. In two unanesthetized rabbits in which the blood pressure was not ascertained, the same injection procedures were successfully followed and with the same effects, as disclosed at autopsy. The alterations in the service rendered to the tissues were profound. The skin and voluntary muscles in general stained almost not at all during the routine 3 minute period after the dye injection (Figs. 1 and 2). The liver and gall bladder on the other hand were intensely and evenly stained and so too with the esophagus and small intestine. The stomach on the other hand was sometimes almost unstained throughout (Fig. 2), and again exhibited serpiginous pallid patches which were or were not limited to the mucosa. The large intestine sometimes showed

patching throughout and again was completely unstained. The omentum was marbled with stain or completely unstained. The spleen was sometimes blue, and again was contracted and not stained. The lymphatics deriving from the stained gut held deep blue fluid, whereas the contents of those from the pallid large bowel was colorless. No great contraction of any of these channels was discernible.

Both the preparations employed caused an interruption of service to the stomach and large bowel as shown by the more or less complete failure of these viscera to stain. The small intestine was by contrast deeply colored. The "Pituitrin" caused vigorous peristalsis with expulsion of feces, whereas the "Infundin" did not elicit this activity, the feces being retained, and the stomach and bowel, examined before life had wholly ceased, showing no abnormal contraction. It is conceivable that in some situations contraction of the muscular layer of the gut may have reinforced vascular contraction, with result in the ischemia encountered. But staining with the dyes employed is so extraordinarily swift that even a temporary relaxation of the muscle, as during peristalsis, would have sufficed to permit it, especially since the blood pressure was abnormally high. The inference is that the ischemia was due to the same cause as that in the skin and muscles, namely to vascular contraction.

It is possible that had the blood volume of the animals been reduced by bleeding just prior to the experiments, or had an amount of pituitrin been given which caused a less intense vascular contraction in the peripheral tissues some diminution in blood service to the small intestine might have come to light. The bloodless state of the skin and muscles has already been commented upon. With the narrowing of the circulatory channels everywhere the blood and the dye added thereto would inevitably be forced to the regions of least resistance, quite irrespective of whether the vessels within these regions,—which might well have been intestinal,—tended to contract. In our experiments the lungs can scarcely have served as reservoirs for blood forced out from elsewhere. They were less stained even than ordinary. Room for some of the excess blood was obtained in the large vessels, as the distended condition of the carotids proved; and some was held in the dilated heart. But in the circumstances of the case, with even the vessels of the stomach and large intestine closed to some extent,

much blood may have been foisted, so to speak, upon the small intestines and liver. Our interest did not lie in determining whether this was actually the case, but merely in the demonstration that the blood vessels in some parts of the gut, at least, are capable of such contraction as to prevent service to the tissues by way of them.

COMMENT

The experiments demonstrate that such vaso-constriction as may take place in the alimentary canal and liver to compensate for a lessened blood bulk never goes so far as to prevent the blood from rendering service to these organs. Not only do diffusible dyes continue to be rapidly distributed to them, but their capillaries fail to offer any obstacle to the passage of india ink particles. In contrast to the state of affairs in the skin and muscles, where well served regions are interspersed amidst others wholly neglected, the service to the hepatic parenchyma and the wall of the gut is evenly distributed. The maintenance of this uniformity was put to an extreme test in the case of the liver by cutting off the venous or arterial blood supply to the organ in animals with blood volume largely reduced. Under these circumstances the remaining stream to the parenchyma flowed with so little force that the slight pressure of ribs and stomach sufficed to turn it aside here and there, with result in patches of total ischemia. Had there been any local differences in the degree of constriction of the small vessels elsewhere in the organ surely these should have been evident in differences in the staining. None were observed, the lobuli being all served to precisely the same extent. Yet the existence of a vaso-motor mechanism within the liver is a fact that cannot be gainsaid. One must suppose it to be so admirably balanced in the healthy animal that all parts of the liver share equally in the blood; for otherwise local hypertrophy, and a concomitant atrophy, would occur (19). Mall observed that a colored mass injected into the freshly extirpated organ reaches the capillaries of the lobules everywhere at the same moment, and this no matter whether introduced by hepatic vein, portal vein or hepatic artery (20). The present experiments prove that the regulation continues undisturbed during the compensatory readjustments which follow reduction of the general or local blood bulk. In view of all these facts the active hyperemia followed by edema

(stroking phenomenon) which Ebbecke elicited on the liver surface must be thought of as a distinctly pathological manifestation.

It had seemed barely possible that under the circumstances of a general depletion the liver lobuli might be intermittently supplied with blood, the rate of intermittence being so rapid that staining with a vital dye or the scattering of india ink to the Kupffer cells would soon appear to have been broadcast. To settle the point thus raised some animals were killed only a few seconds after injection of the test material. Even in them the findings showed an even distribution of blood to the individual lobuli, though within these units one could see that the cells first reached by the stain or ink particles, namely those at the lobular periphery, shared in these substances more abundantly than those nearer the central vein. In the case of normal animals receiving ink,—which substance can be followed with special ease,—this difference disappeared after a few minutes, the Kupffer cells all appearing to have shared equally in the particulate matter, even when the amount was small. So too with cats which had been bled. In bled rabbits, on the contrary, there was a startling change in the distribution, almost all the ink particles being held in the peripheral Kupffer cells, which were swollen and black with the engorged material. A number of reasons for this suggest themselves, amongst them that of the known differences in the intrahepatic vascular responses of the rabbit and cat (21).

The maintenance of a well distributed and effective blood service to the gastro-intestinal tract after hemorrhage is necessary if the gut is to function in aid of recovery. Conditions would be doubly disastrous were service greatly cut down. Yet in view of all that has been written on intestinal blanching and compensatory vaso-constriction within the portal system after hemorrhage one might have expected some regions of local ischemia at the least. In this connection the demonstration of a previous paper may be recalled, that blood service is often still going on in tissues which, to the eye, appear wholly bloodless.

Bayliss has made the generalization that the vaso-motors to the viscera are predominantly vaso-constrictor in type, and those to the periphery vaso-dilator. While this generalization holds for the ordinary circumstances of life, certainly when the blood bulk is reduced the compensatory vaso-constriction is far more effective at the periphery than in the liver and alimentary canal.

SUMMARY

The vascular readjustments in compensation for a greatly reduced blood bulk affect the service rendered by the blood to the gastro-intestinal tract and liver far less than they do that to the skin and muscles. Into these latter tissues india ink is carried almost not at all, whereas it circulates in quantity through the capillaries of the bowel and liver. Evidently vaso-constriction is much less effective in these viscera. Nowhere in them does one find a patchy ischemia like that so wide-spread in the peripheral tissues. Blood service is maintained to the same extent everywhere throughout the liver even when one of its two sources (hepatic artery or portal vein) is obstructed, and the intrahepatic blood pressure brought very low.

A pronounced patchy ischemia of the stomach and large bowel can be induced by intravenous injection into normal animals of sufficient epinephrin to cause the systemic blood pressure to mount to an abnormally high level. Pituitrin used in the same way has a greater effect; blood service to the organs mentioned may be completely abolished by means of it. In both instances, though, service to the small gut and liver is still excellently and evenly maintained.

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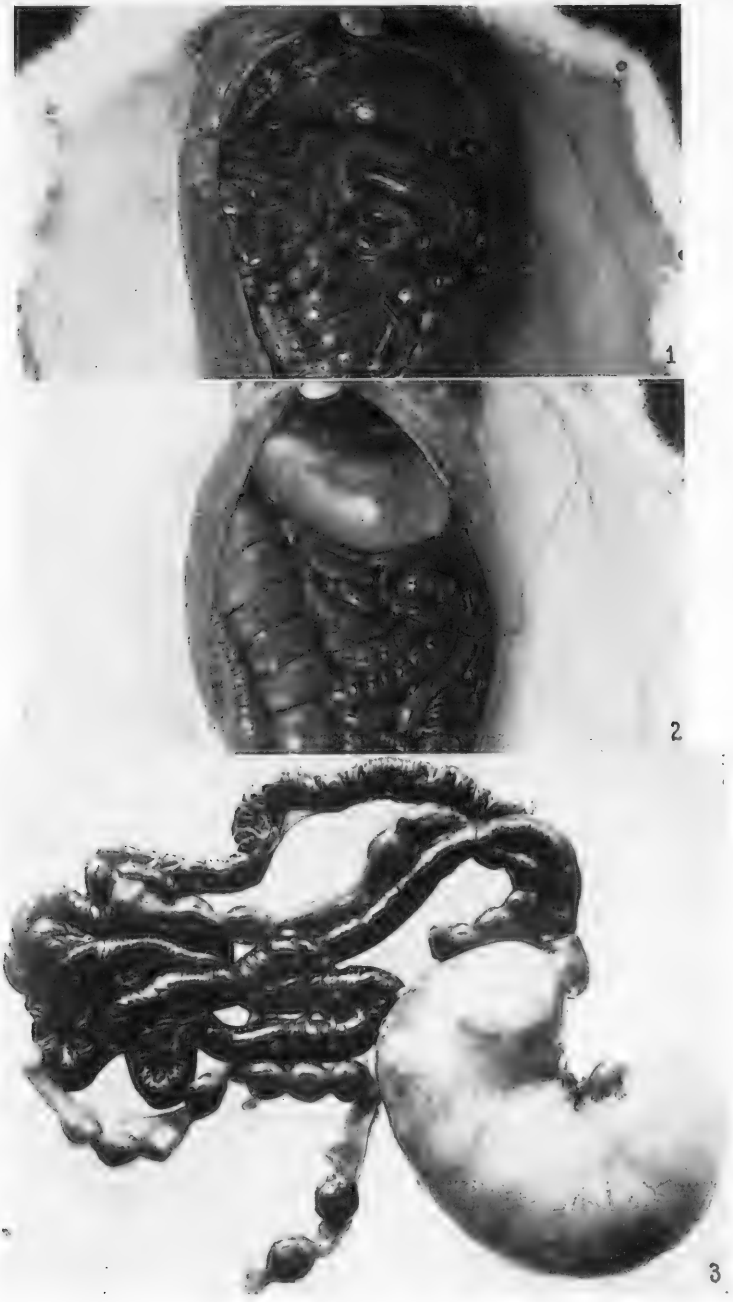
EXPLANATION OF PLATE 9

Fig. 1. Organs of an unanesthetized white rabbit receiving brom phenol blue intravenously and decapitated two minutes later.

Fig. 2. Organs of a white rabbit treated in the same way but receiving pituitrin four minutes prior to the stain.

It will be seen that the skin of the right side of the pituitrin animal is unstained whereas that of the control was colored, and though the subcutaneous muscle (seen attached to the skin on the left side) was stained that of the control was much more deeply so. The stomach of the pituitrin rabbit though mottled with dye was largely unstained, whereas the stomach of the control was intensely colored. The small intestines of both animals were stained to the same great degree, but the caecum of the pituitrin animal showed some splotchy, partial pallor. The blue of the animals photographed so poorly that the brilliant character of the differences is far from evident.

Fig. 3. A more pronounced instance of pituitrin ischemia. The stomach of the rabbit injected with pituitrin followed by brom phenol blue has stained almost not at all, and so too with the descending colon,—which is recognizable by its content of fecal pellets. The small intestine, on the other hand, is intensely colored.



(Gilding: Studies of tissue maintenance. II)

THE EFFECT OF UNILATERAL NEPHRECTOMY ON THE TOTAL NUMBER OF OPEN GLOMERULI IN THE RABBIT

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Plates 10 and 11

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Karsner, Bunker and Grabfield (1) and others have demonstrated that removal of one-half the kidney substance in the dog results in a transient renal insufficiency with complete recovery in 1 to 2 days. It is apparent that the opposite kidney compensates for the loss. The majority of the published papers summarized by Hinman (2), deal with blood and urine findings and late hypertrophic changes. So far as we are aware, there is no work to explain the immediate compensation occurring after unilateral nephrectomy.

Through the work of Richards and his collaborators (3) the conception of periodic functioning of glomeruli has been placed on a firm foundation. Hayman and Starr (4) have demonstrated that in general, kidney volume, renal blood flow and urine elimination vary in direct proportion to the total number of open glomeruli.

Method

The technique of Hayman and Starr (4) with some modifications was followed. Rabbits were used throughout. As indicated in the protocols ether anesthesia was employed in some, and in others section of the lumbar spine by the technique of Ecker (5).

In control animals the lower abdomen was opened, the intestines wrapped in a warm towel, the aorta isolated and clamped at the bifurcation and just below the renal vessels, a long slender glass cannula introduced into the aorta between the clamps, the upper clamp removed and the cannula inserted so its tip was at the level of the diaphragm. The outside diameter of the cannula was approximately

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one half of the internal diameter of the aorta. With the cannula in position, the superior mesenteric artery and coeliac axis were clamped and 0.75 cc. of 3 per cent solution of Janus Green B (Coleman and Bell) in 0.9 per cent NaCl solution injected under moderate pressure. The time elapsed during the injection averaged 5 seconds. Within 10 seconds the left renal artery was ligated and the animal killed by a blow on the head or by ether. In some cases the left renal circulation was irrigated with saline and filled with 5 per cent ammonium molybdate. In others the left kidney was removed, cut in thin slices and placed in the molybdate solution. After death of the animal the right kidney was injected by the method of Nelson (6).

In experimental animals, the right kidney with as long a pedicle as possible was removed through a lumbar incision. It was then injected supravitally with Janus Green for a determination of the total number of glomeruli. After a variable period, the left kidney was stained intravitally by the procedure outlined above for control animals.

In the animals on which a sham operation was performed, the kidney region was exposed by lumbar incision, the kidney freed from its bed, the pedicle exposed, the whole replaced and in some cases a suspension suture placed in the capsule. After variable periods these animals were subjected to the same procedure of intravital staining as the control and experimental group.

The whole procedure may be summed up as follows: One kidney is injected supravitally to determine the total number of glomeruli in one kidney of this animal; the opposite kidney is injected intravitally for a determination of the number of glomeruli which are in active circulation during a period of 5 seconds under certain experimental conditions; and division of the latter by the former gives the percentage of open glomeruli.

The counts were made after the general technique of Vimtrup (7). The cortex and medulla are separated and the weight of the entire cortex secured. From the cortex, 6 to 10 small samples are removed, pooled and accurately weighed. The total weight of these samples varied from one-tenth to one-sixth of the entire cortex. From the number of glomeruli in these samples of known weight, the total is secured by estimation. In some cases, microscopic sections were also prepared to determine the completeness of injection in supravital injections and to check the percentage injected by the intravital method.

Results

As shown in Table I, in seven adult rabbits, the percentage of open glomeruli varied from 44 per cent to 78 per cent with an average of 63 per cent. In general those in which ether was used as an anesthetic have slightly more than those with fractured spine anesthesia.

After unilateral nephrectomy the percentage was increased to 91 to 99 per cent with an average of 95 per cent (Table II). This

increase appeared in less than 24 hours and continued until at least 10 days after nephrectomy.

TABLE I

Normal Controls

Rabbit No.	Supravital		Intravital		Per cent open	Anesthetic
	Kidney No.	Count	Kidney No.	Count		
1	45	179,767	46	79,640	44	Section spine
2	51	185,769	50	101,193	54	Section spine
3	69	158,400	70	124,410	78	Ether
4	71	147,734	72	110,670	74	Section spine
5	76	152,238	75	100,702	67	Section spine
6	78	138,406	77	92,386	67	Section spine
7	80	149,134	79	98,210	66	Section spine
Average.....					63.5	

TABLE II

Experimental

Rabbit No.	Supravital		Interval in days after nephrectomy	Intravital		Per cent open
	Kidney No.	Count		Kidney No.	Count	
8	93	102,300	1	98	99,700	97
9	94	132,750	2	99	130,111	98
10	95	170,930	3	100	165,453	92
11	96	135,670	5	101	122,500	90
12	97	181,000	10	102	178,420	98
13*	54	87,350	1	56	80,222	92
14*	55	111,508	2	58	101,538	91
15	61	172,331	3	63	169,210	98
16*	57	68,620	5	60	67,752	98
17*	62	96,250	10	64	95,499	99
18	126	150,364	1	106	156,217	100
19	127	154,782	2	107	149,167	97

* Young animals, 5 to 7 months of age; remainder over 1 year of age.

The animals subjected to a sham operation (Table III) do not differ markedly from the normal controls. The open glomeruli vary from 51 per cent to 75 per cent with an average of 64 per cent.

DISCUSSION

There are certain points in the technique which need further comment, in order to validate the results.

Janus Green B is known to be a powerful vaso-constrictor and it is possible that it might cause such constriction that the results are not those present in life. Hayman and Starr (4) used India ink and obtained the same figures. Further, their results with salt and with caffeine, as well as the results here described following unilateral nephrectomy, prove that 90 to 100 per cent may be open with the same experimental technique.

TABLE III

Sham Operation

Rabbit No.	Supravital		Interval in days after operation	Intravital		Per cent open
	Kidney No.	Count		Kidney No.	Count	
23	118	161,208	1	119	82,344	51
24	104	150,100	1	103	102,614	68
25	120	158,316	2	121	93,562	59
26	122	122,488	5	123	86,920	71
27	124	135,728	10	125	100,786	75
Average						64

The percentage figures depend on the assumption that the two kidneys of any one animal each contain approximately the same number of glomeruli. In addition to the proof offered by Haymann and Starr we add the results in two rabbits.

Rabbit No.	*Left*	*Right*
30	145,200	152,328
31	165,160	160,209

We also have unpublished observations that this is true in man and rats. The variation is rarely over 5 per cent. Further proof is adduced by the agreement between the counting method and the study of sections with determination of the relative number stained.

The accuracy of the weight estimation method for determination of the total number of glomeruli may be open to some doubt. We be-

lieve it to be accurate to within 10 per cent. Vimtrup (7) has offered evidence to support this view. Work soon to be published from this laboratory on the total number of glomeruli in the kidney of man and animals will further support the accuracy of the method.

The distribution of the dye in intravitally stained normal kidneys is peculiar. Never are the stained glomeruli distributed evenly throughout the cortex. As noted by Hayman and Starr, often the peripheral glomeruli are not stained while the more central are all stained. In other cases there are small to large areas corresponding to the distribution of an interlobular or interlobar artery in which very few glomeruli are stained. In selecting the specimens for counting, such areas must be taken into consideration and a proportionate amount of them contributed to the pooled specimen.

Richards and Schmidt (3) have noted that an increase in the number of open glomeruli is associated with an increase in the number of open loops of any one glomerulus. In general, we have found this to be true (compare Fig. 3 and Fig. 4). In the active kidney, more glomerular loops are stained and those that are stained are more definite and larger.

The results themselves are unequivocal and need little discussion. The results on the sham operation animals prove that the operation itself is not the cause of the increase. Experiments are under way to contribute evidence on the cause of the increase.

SUMMARY

1. Under the experimental conditions employed, from 44 to 78 per cent of the glomeruli of the normal rabbit kidney contain circulating blood at any one moment.

2. After unilateral nephrectomy the number of glomeruli in the remaining kidney, which contain circulating blood, is increased to 91 to 99 per cent.

3. Compensation for the removal of one kidney is accomplished during the first 10 days at least, by an increase of the number of open glomeruli in the opposite kidney.

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EXPLANATION OF PLATES

PLATE 10

FIG. 1. A teased preparation of cortex injected by Janus Green B, to show the definition of the glomeruli. Such preparations are used in counting. × 8.5.

FIG. 2. Section of kidney supravitally stained. × 110.

PLATE 11

FIG. 3. Section of normal kidney intravitally stained with Janus Green B. Note the unstained and partially stained glomeruli. Compare with Fig. 4. × 90.

FIG. 4. Section of kidney from animal after unilateral nephrectomy intravitally stained with Janus Green B. Note the absence of unstained glomeruli and the more complete staining of the glomerular loops. Compare with Fig. 3. × 90.

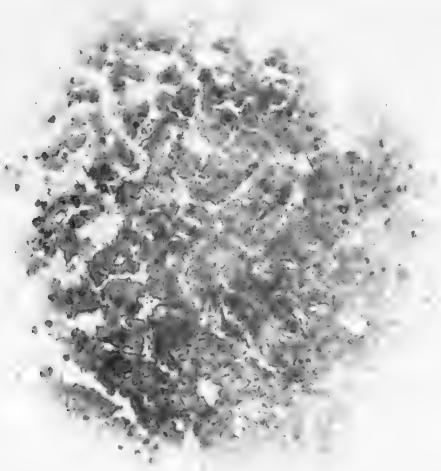

FIG. 1

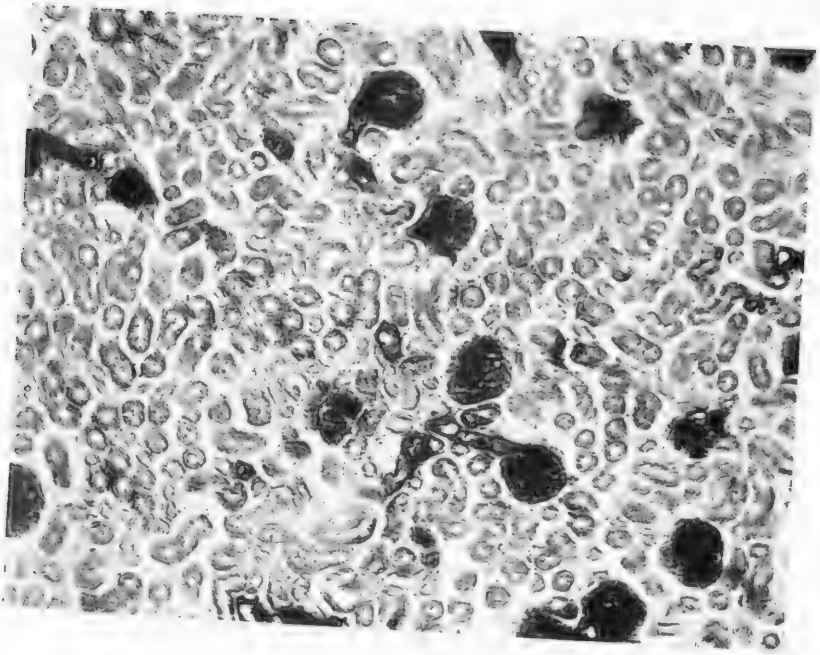

FIG. 2

(Moore and Lukianoff: Unilateral nephrectomy in the rabbit)

UNIV OF

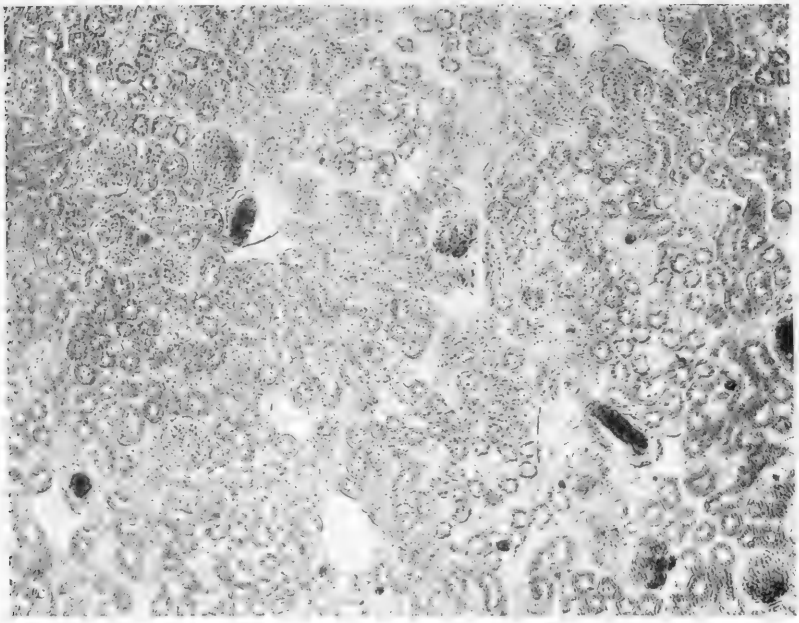

FIG. 3

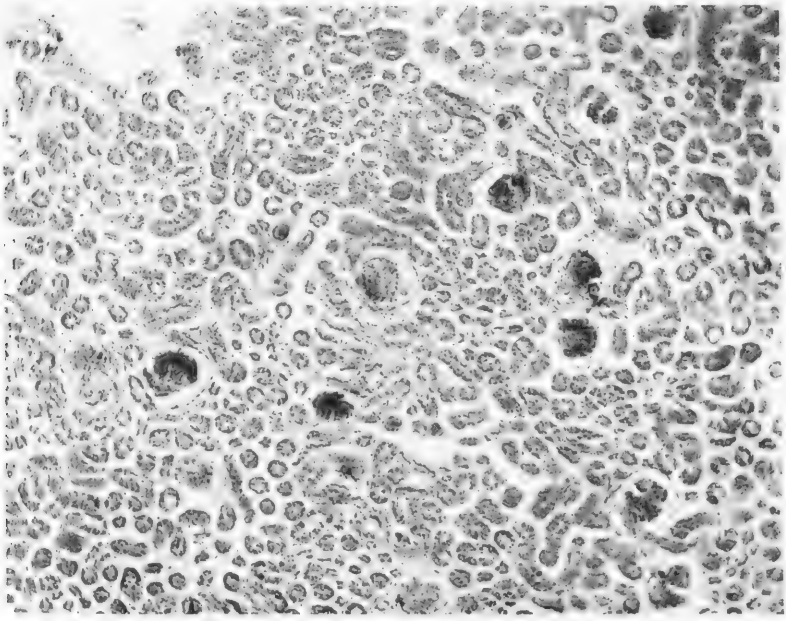

FIG. 4

(Moore and Lukianoff: Unilateral nephrectomy in the rabbit)

A STUDY OF PNEUMONIA IN A RURAL AREA IN SOUTHERN ALABAMA

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(Received for publication, April 4, 1929)

The present study was undertaken to determine certain basic facts in relation to pneumonia in a rural community in the South; it covers a period of 6 months, October to April, 1927–1928. Our field laboratory[1] was established in Andalusia, Alabama, a town of about 4,000 inhabitants, the center of a rather sparsely settled rural community where farming and lumbering are the chief industries. The area covered in the study comprises approximately a thousand square miles, and has a population of about 35,000, the great majority of whom are white.

Types of Pneumococci Isolated

Fifty-eight cases of pneumonia were seen in the Andalusia area during the course of the study. A summary showing the types of pneumococci isolated from these cases is given in Table I.

The striking feature of this series of cases is the small number of fixed types of pneumococci that were isolated. Pneumococci of the heterogeneous Type IV group were the prevailing organisms.

Severity of the Disease

In Table II we have classified the cases of pneumonias according to their severity as correlated with the type of pneumococcus isolated.

Lobar pneumonia takes a much heavier toll in the northern than in the southern United States. In 1925, for example, the death rates

[1] We are greatly indebted to Col. C. A. Reasoner of the U. S. Army Medical Service, for help and advice in selecting equipment for our field laboratory.

per 100,000 from all types of pneumonia in whites and negroes for certain typical states were as follows:

	White	*Colored*
New York	105.3	367.4
Massachusetts	116.4	230.5
Pennsylvania	116.7	366.4
Alabama	81.4	135.1

TABLE I

Types of Pneumococci Isolated Compared with Data from New York City (Monograph 7, Rock. Inst.)

Pneumococcus Type	No. of cases	Alabama	New York City
		per cent	*per cent*
I	7	12.3	33
II	0	0.0	31
IIx	2	3.5	—
III	1	1.7	12
IV	40	68.5	24
Pneumococcus not isolated	8*	14.0	—

* Two of this group had a pneumonia due to staphylococcus, one following a periostitis—the other a case of senile dementia, while one case had a hemolytic streptococcus in pneumonia following an automobile accident. The remaining five were children from 1 to 8 years of age, from whom it was difficult to get sputum. They were presumably pneumococcus pneumonias, Type IV.

TABLE II

Severity of the Disease

Type	Mild	Moderate	Severe	Fatal
I	0	3	4	0
IIx	0	0	0	2
III	0	0	1	0
IV	21	11	6	2
Pneumococcus not isolated	4	1	1	2*
	25	15	12	6

* Staphylococcus pneumonia.

Since no morbidity data are available, it is not certain whether pneumonia is less prevalent in the south than in northern states or equally prevalent but less fatal.

Tables I and II suggest that pneumococcus pneumonia may be as frequent in the isolated rural districts in the south as in the large cities in the north, but is much less fatal.

Age Distribution

In Table III we have summarized the distribution of cases of pneumonia by age groups. The striking feature of Table III is that pneumonia due to Type IV pneumococcus occurred chiefly in children under 15 years of age, whereas pneumonias due to the fixed types of pneumococci were seen chiefly in adults.

TABLE III

Age Distribution of Cases of Pneumonia in Southern Alabama

Age	Total cases	Fixed types of pneumococci	Type IV	Pneumococcus not isolated
Under 1 year	1	0	1	0
1– 4 years	9	0	6	3
5–14 "	26	4	19	3
15–44 "	15	4	10	1
45–64 "	4	2	2	0
65 and over	3	0	2	1
	58	10	40	8

Seasonal Distribution of Pneumonia

The community studied was free to a great extent from acute respiratory disease until the first week in January, though there had been mild outbreaks of "colds" in neighboring counties. Daily records were made of maximum and minimum temperature, relative humidity and rainfall. The exact day of onset of each case of pneumonia was recorded. Directly following a week of low temperature, Jan. 1, there occurred a widespread epidemic of colds. Aerobic cultures from the nasopharynx of many individuals during this outbreak showed (a) a great preponderance of pneumococci Type IV, of low virulence to white mice, (b) a large number of influenza bacilli of various types. The time relationship between the cold weather and the prevalence of pneumonia is shown in Chart I. The data seem to

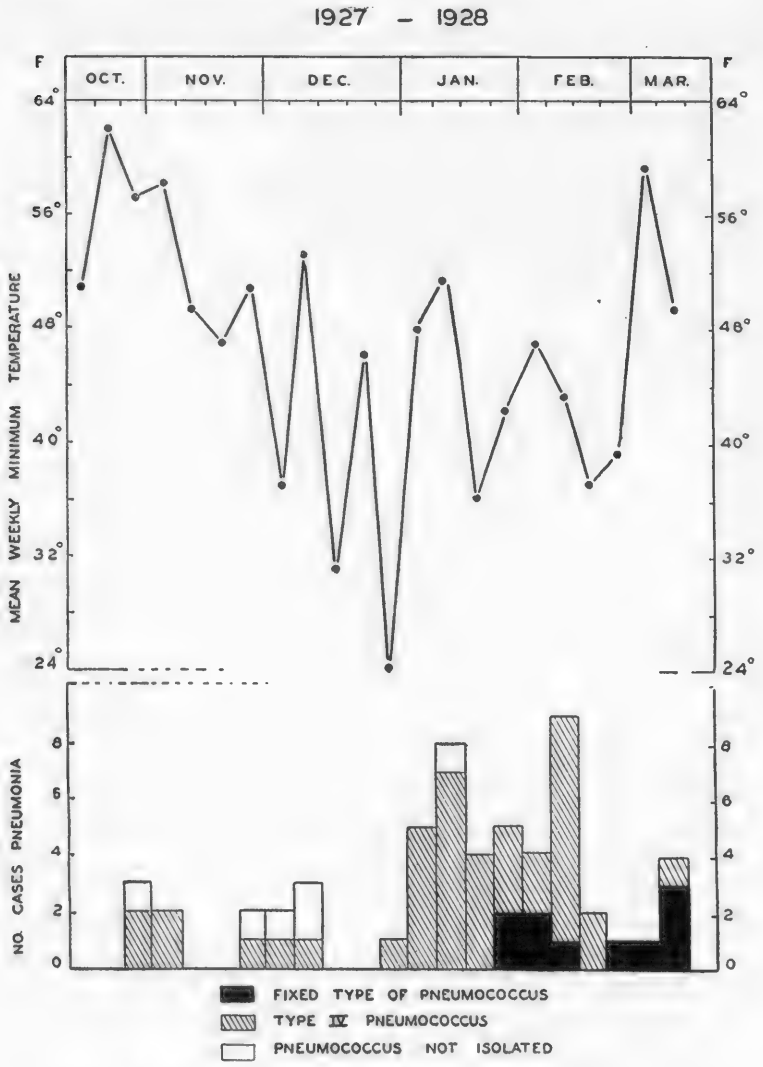

CHART I. Seasonal distribution of pneumonia, Andalusia, Alabama.

indicate that a period of cold weather with attendant suffering and exposure bore a definite relationship to the onset of pneumonia. The sequence was first, cold weather; second, an epidemic of colds; third, pneumonia. The cases of pneumonia did not occur during the coldest weather but 2 to 4 weeks after the weather became warmer.

In individual cases the acute colds developed into pneumonia, usually on the sixth or seventh day of the cold, often following exposure while riding several hours in a wind, or getting rain-soaked and thoroughly chilled when returning from school. The great proportion of the cases occurred in children under 15 years of age and the invading pneumococcus was usually Type IV. Frequently two or more members of the family developed pneumonia at the same time.

TABLE IV

Relationship of Exposure and of Acute Colds to Pneumonia

	Type of pneumococcus			
	Fixed types	Type IV	Pneumococcus not isolated	
Sudden onset—no exposure or "acute cold"	2	4	2	
Exposure but no "cold"	1	1	0	Total
	3	5	2	10
"Acute cold" antedating pneumonia 5–10 days	6	25	3	
"Acute cold" plus exposure to chilling rain, etc.	1	10	3	Total
	7	35	6	48

The relationship of the onset of pneumonia to chilling and exposure and also to acute colds is given in Table IV.

The clinical symptoms in these cases of Type IV pneumonia were typical of lobar pneumonia, but the physical signs were not characteristic of those found in cases of lobar pneumonia due to the fixed and more virulent types of pneumococcus.

The signs of consolidation in the lung were typical but the area involved did not conform to the anatomical outlines of a single lobe. Often areas of consolidation in two different lobes in the same lung were found—sometimes both lungs were affected. The cases could not be called broncho-pneumonia, for small multiple areas of con-

solidation were not found, but rather large areas with typical massive consolidation, sometimes in the axilla but most often in the right base.

The epidemiology of these cases of pneumonia in a sparsely settled rural area is in direct contrast to pneumonia in our larger northern cities where adults are more commonly affected than children and family epidemics are rare; where the attack usually runs a severe course, may or may not be preceded by a cold, and where the physical signs are usually confined sharply to the anatomical outline of a lobe of lung.

It seems a tenable supposition that the isolation, poor roads, lack of frequent contact with one another and with the outside world, have produced in a community of people a low resistance to relatively avirulent pneumococci, whereas under more crowded conditions, there develops a community resistance to avirulent pneumococci, where pneumonia occurs as a rule following an invasion of one of the virulent fixed types of pneumococci.

The mode of life, customs, and habitations of the community studied are closely comparable with those of our forefathers one hundred years ago or more. It is interesting to note that the medical literature of 1810 to 1840 is full of references to epidemic pneumonia.

Warner (1), 1814, describes the epidemic prevailing in New England and New York State. He states "the disease is characterized by an initial chill and prostration and seems related to exposure to wet or cold." Cartwright (2) describes an epidemic in Natchez in 1826 and notes that atmospheric vicissitudes, exposure to inclement weather and intemperance predispose to the disease. MacBride (3), 1813, in describing an epidemic in St. John's Parish, South Carolina, states that most of the deaths occurred in field negroes, but the disease was also seen in whites of the lower classes. When an individual in a family was attacked, nearly all other members developed the disease. McCall (4), 1823, describes a family epidemic in Kentucky; Williamson (5) described an epidemic in 1813 in North Carolina and felt that cold and rain predisposed to the disease. Smith (6), Stearns (7), Mott (8), Mann (9), Eights (10), LeComte (11) and many others describe epidemics of lobar pneumonia in various parts of the eastern United States in the early part of the last century.

It seems probable that the pneumonia in the isolated rural community studied is comparable to the type of pneumonia of pioneer days in the United States, and that the increase of population and the

industrialization of the northern states, producing crowded conditions and frequent contacts, have resulted in the development of a population largely immune to avirulent strains of pneumococci, but responding to certain of the fixed virulent strains.

Family Epidemics of Pneumonia

In seven different families two or more members of the family were ill with pneumonia at the same time. This is contrary to the usual finding in the northern United States, where family epidemics of pneumonia are rare.

The intimate relationship of one case of pneumonia with another suggested the possibility of transmission of the disease from one member of a family to another by immediate contact. If this were the case, then one would expect to find that the various contacts with a case of pneumonia would harbor in their nasopharynx the type of pneumococcus corresponding to that found in the sputum of the patient. To determine this point, a study was made of the nasopharyngeal flora of 26 families in which one or more cases of pneumonia had developed.

Technique

Practically every case of lobar pneumonia which occurred in the area during the winter was seen in consultation with the family physician[2] as soon as the diagnosis of pneumonia was made (usually the second day of the disease). Cultures of the nasopharynx of all contacts were made at once, using the West tube. They were placed directly in blood broth, kept warm during transportation, and plated on 3% horse blood hormone agar plates as soon as possible. The plates were searched after 24 to 36 hours, and typical colonies were isolated and the organism identified. A total of seventy-three contacts were cultured, each of whom had been in close and frequent communication with the pneumonia patient. A summary of the findings is given in Table V.

It might be assumed from Table V that the thirty-two contacts with cases of Type IV pneumococcus who harbored type IV strains in their nasopharynx were infected by actual contact with the patient who was ill with pneumonia in the household. If this were the case, contacts with Type I and Type II pneumonias should harbor corresponding strains. This occurred in only five instances, whereas ten of

[2] There are about twenty-five general practitioners in the area.

the seventeen contacts with Type I pneumonia harbored not Type I but Type IV pneumococcus. It seemed probable, therefore, that some other factor than contact with a case of pneumonia was responsible for the prevalence of pneumococci in the nasopharynx of these individuals.

TABLE V

Prevalence of the Pneumococcus in the Nasopharynx of Individuals in Direct Contact with Pneumonia Patients

	Type of pneumonia in patients to whom contacts were exposed		
	Type I	Type II	Type IV
Contacts harboring pneumococcus:			
Type I	2*	0	0
Type II	0	3	0
Type III	0	1	0
Type IV	10	1	32
No pneumococci found	5	2	17

* Both these contacts developed Type I pneumonia within 3 days after their throat cultures were taken.

TABLE VI

Correlation of the Prevalence of Pneumococci in the Nasopharynx with an Acute Respiratory Infection

Type of pneumococcus found	Acute cold when examined	Just recovered from cold	No history of recent cold
I	2*	0	0
II	1†	0	2†
III	0	0	1†
IV	25	15	3
No pneumococcus found	6	13	5
Total	34	28	11

* Contact with Type I pneumonia.
† Contact with Type II pneumonia.

We have noted that practically all cases of pneumonia were preceded by an acute upper respiratory infection and that these colds occurred as family epidemics affecting all members. It seemed possible that these epidemic colds might be associated with an in-

creased prevalence of pneumococci. An analysis was made, therefore, to determine this point. See Table VI.

Table VI shows clearly that a large proportion of the individuals in this study who were harboring pneumococci had colds at the time or had just recovered from them. This is particularly true of the group of individuals who made up the families in which there was a case of Type IV pneumonia. Furthermore, the relative number of pneumococcus colonies on the blood agar plate cultures of the nasophaynx seemed to bear some relationship to the course of the cold. See Table VII.

Tables VI and VII give a very definite indication as to what occurred in these epidemics of colds and the relationship of the cold to pneu-

TABLE VII

Group of Contacts with Type IV Pneumonia; Table Showing Relative Prevalence of Pneumococci Type IV in Acute "Colds"

Proportion of pneumococcus colonies on the blood agar plate in relation to all other colonies	Number of cases			
	1st 3 days of cold	4th to 8th day	1st week after recovery	2nd week after recovery
50 to 100 per cent	1	5	3	0
25 to 50 per cent	0	3	5	1
Few (less than 25 per cent)	5	3	1	2
No pneumococci	2	3	3	7

monia. Within the first few days of the cold, pneumococci Type IV are found in the nasopharynx of those affected. From the fourth to the eighth day, the pneumococci have so increased as to outnumber all other organisms. As the patient recovers from the cold, the pneumococci disappear and the normal flora of the throat reappear, though pneumococci are frequently found for 10 days to 2 weeks after symptoms of cold have disappeared.

It seems probable that the contacts with cases of pneumonia, particularly the Type IV group, were not infected by their direct contact with a pneumonia patient. The more probable history is as follows: An epidemic of acute colds occurred in a family, usually affecting all members of the family. Coincident with the cold, pneumococci occurred in the nasopharynx in large numbers. In the great majority

of the colds, nasopharyngeal symptoms disappeared after 7 to 10 days, and gradually the pneumococci disappeared also. A small proportion of the individuals who had an infection with pneumoccci of the upper respiratory tract developed pneumonia on the fourth to eighth day of their cold. The attack often followed an exposure or chilling of the body surface, with a resultant extension of the infection from the upper to the lower respiratory tract. The pneumonia, therefore, was an incident in the course of an epidemic of colds.

If this is true, family epidemics of colds associated with pneumococci must have occurred in which no case of pneumonia developed. Such indeed was found to be the case. A complete epidemic of acute colds was studied in a small rural school—cultures being taken before the colds began, during the epidemic, and after the epidemic had ceased. A report of this epidemic, together with other similar studies, will be made in a subsequent communication.

The assumption might be made that the incitant of these colds is the pneumococcus—especially since there is some contributory evidence in addition to that already presented. One bit of evidence is that these colds were associated with a high leucocytosis—a white blood count of twelve to fifteen or even eighteen thousand being commonly found. Furthermore, one of the present investigators developed a clear-cut nasopharyngeal infection due to pneumococci which was acquired in the laboratory while working with the pneumococcus cultures and which ran a course similar to the family epidemics of colds. The appearance and disappearance of symptoms in this infection were closely correlated with the appearance and disappearance of pneumococci in the nasopharynx.

In one instance, a pure culture of pneumococci Type I was found in the nasopharynx on the second day of a cold in a person who was nursing a patient with Type I pneumonia. 3 days later the nurse developed pneumonia Type I. In two other instances mothers who were caring for children with pneumonia Type IV developed typical colds, and almost pure cultures of pneumococci Type IV were isolated from the nasopharynx on the first or second day of the cold.

But there is evidence also that pneumococci were merely contributory causes and perhaps did not initiate the colds at all. It will be noted from Table VII that pneumococci were not abundant in the

nasopharynx during the first 2 or 3 days of the cold, but were most prevalent from the fourth to the eighth day. This observation suggests that the pneumococci may be secondary invaders and that the cold was initiated by some unknown factor.

Another interesting observation in connection with these family epidemics of colds associated with pneumococci is that Pfeiffer bacilli were frequently found in abundance in association with the pneumocci. In general these organisms appeared later in the cold than the pneumococcus, and were present for a longer period after symptoms had disappeared. No constant type of Pfeiffer bacilli was encountered. Both hemolytic and non-hemolytic forms were found—

TABLE VIII

Table of Correlation between Incidence of "Colds" and Prevalence of Pneumococci and Pfeiffer Bacilli in the Nasopharynx

	Number of cases			
	Acute cold at present	Just recovered from cold	No history of recent cold	Total
Pfeiffer bacilli and pneumococci	11	6	0	17
Pfeiffer bacilli only	4	8	1	13
Pneumococci only	15	11	5	31
Neither pneumococci nor Pfeiffer bacilli	5	3	6	14

some requiring V + X factors for growth, others requiring only V factor. Some produced indol—others did not. There was some consistence in the type found in members of the same family but not in the group as a whole. A brief summary of these findings is given in Table VIII.

Table VIII indicates that Pfeiffer bacilli were frequently associated with pneumococci in the acute colds which occurred in the group of contacts with cases of pneumonia.

CONCLUSIONS

1. Pneumococcus Type IV of low virulence was the prevailing organism in fifty-eight cases of pneumonia studied in southern Alabama. Fixed types of pneumococci were not common.

2. Pneumonia was more prevalent in children from 5 to 15 years of age than in adults. As a rule, the disease ran a mild course.

3. Most of the cases of pneumonia gave a definite history of an acute cold antedating the attack of pneumonia by a period of 5 to 8 days. Exposure alone did not seem to predispose to pneumonia, but those with an acute cold who were exposed to chilling of the body surface frequently developed pneumonia.

4. There were seven "family epidemics" of pneumonia. In each instance there was a family epidemic of colds antedating the pneumonia. The pneumococcus was found in large numbers in the nasopharynx of those suffering from colds as well as in the pneumonia patients.

5. The epidemiology of pneumonia in the pioneer days of American history has many points in common with the epidemiology of pneumonia in a rural isolated area in southern Alabama today. This suggests that the crowded conditions and frequent contacts of modern city life have built up a community resistance to avirulent strains of pneumococci.

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STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

I. The Isolation and Differentiation of Dissociants

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Plate 12

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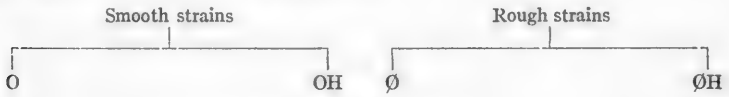
The question whether the dissociation occurring in the paratyphoid group of bacteria follows the H and O type of dissociation of Weil and Felix, the S and R type of Arkwright or a combination of both is still unsettled.

According to Hadley (1) (p. 141) the somatic and flagellar antigen hypothesis of Malvoz should be abandoned since certain nonflagellated bacteria split into 2 forms, R and S, which give the same order of serological reactions as those observed in the R and S types of motile bacteria.

Arkwright and Goyle (2) claimed that the O and H antigens were essentially those of smoothness and roughness. This view was however criticized by White (3) who has represented the variation of the paratyphoid group in a scheme which in a somewhat simplified form is as follows:



O = smooth, heat stable, granular antigen complex.
H = heat labile, floccular antigen complex.
Ø = rough, heat stable, granular antigen complex.

He has also pointed out that the flagellar and somatic agglutinins were identical with the H and O antigens, and that motility was entirely restricted to bacilli giving definite H reactions. No members of the O variety were motile. There are theoretically, therefore, disregarding the specific and nonspecific dissociation of Andrewes (4), and aside from the normal motile smooth (OH) form, three possible forms of variants: the nonmotile smooth (O), the motile rough (ØH), and the nonmotile rough (Ø). Arkwright (5) has demonstrated the existence of these variant forms of *B. paratyphosus* A.

It is obvious that the dissociation phenomenon of the motile paratyphoid group of organisms is more complex than that of nonflagellated forms like *B. dysenteriae*. The simple S and R conception of dissociation does not seem to be adequate to account for the several variant forms encountered among the flagellated bacteria.

Smith and Reagh (6) first reported the existence of both motile and nonmotile strains in hog cholera bacillus and demonstrated the difference between flagellar and somatic agglutinins. Their work was soon confirmed by Beyer and Reagh (7), Orcutt (8) and others.

In the present investigation observations on the dissociation of the hog cholera bacillus were made by using a special semi-solid medium. Three distinct variant forms arising from the original "normal" strain were observed.

EXPERIMENTAL

Hog cholera bacillus XVI obtained from The Rockefeller Institute, Princeton, N. J., was used. The bacteria derived from the characteristic smooth colonies were actively motile and showed the normal biochemical and serological reactions for this type. Single colonies of the motile smooth (MS) form were selected from 3 successive platings to obtain a pure line culture. This culture was then inoculated in meat infusion broth, incubated at 37°C. for several weeks, and plated in semi-solid medium for study.

The semi-solid medium described by Hiss (9) was used. This medium was found to be useful not only for the isolation of the usual motile and nonmotile forms but also for the differentiation of other variants. The medium contains agar 10 gm., gelatine 160 gm. and meat infusion bouillon 2000 cc. The agar was dissolved in 600 cc. of distilled water by boiling for 20 to 30 minutes. Bouillon was added and the boiling continued for about 10 minutes, during which time, the gelatine was slowly added. Water was added to make up the volume lost by evaporation. The mixture was adjusted to pH 7.4, sterilized in the autoclave for 20 minutes, cleared by sedimentation in an inspissator, and filtered through absorbent cotton at 45°C. The medium was then tubed while hot into large test tubes each containing 15 cc.

This semi-solid medium was inoculated with 0.2 cc. of a suspension of hog cholera bacilli made by adding one loopful of the old broth culture to 30 cc. of normal saline solution, and plates were poured. The medium formed a layer approximately 5 mm. thick. The plates were incubated at 37°C. for 15 to 18 hours and were placed in the refrigerator for about an hour to congeal the medium. They were then examined by holding them above and in front of a Leitz microscope lamp or under the low power lens of a microscope.

Dissociation Forms Encountered

Thus far in the study of colony variation of the typhoid-paratyphoid group, the surface colonies on agar plates have been observed and little attention has been paid to the deep colonies growing in semi-solid medium. In the present study, after the broth culture had been kept at 37°C. for about 3 weeks and plated, only the deep colonies which grew in the semi-solid medium were studied. The following forms of deep colonies were observed:

1. Homogeneous Colonies (Motile Smooth).—These were large, round, disk-like, transparent colonies with smooth margins and surfaces,—the "normal" type form (Fig. 1). The center was as a rule more dense than the periphery, and occasionally appeared as an ill-defined spot. Concentric zones of varying thickness which appeared to be the zones of migration were usually present. Colonies 18 hours old generally measured from 2 to 3 cm. in diameter.

The individual organisms of this form were well-separated and actively motile. They grew in a homogeneous cloud in broth. The growth from an agar slant formed a diffuse suspension when placed in normal saline solution. When grown on the surface of an agar plate, these bacteria formed smooth, round colonies.

This form was designated MS (motile smooth).

2. Irregular Colonies (Motile Rough).—These forms were irregularly shaped, and consisted of clumps or clusters of small secondary colonies (Fig. 2). The characteristics were best observed under low power magnification. Some of the colonies were more or less disk-shaped but the ill-defined margins and surfaces and the presence of clumps of secondary colonies differentiated them from the homogeneous (MS) type. These colonies were much smaller than the homogeneous form, generally measuring from 1 to 3 mm. in diameter.

The bacteria from this form of colony occasionally formed short chains and clumped together. Some of the clumps moved sluggishly as units. Actively motile, individual bacilli were also present. In broth cultures the growth clumped, settled to the bottom and left a clear supernatant fluid. Bacteria from an agar slant culture, when suspended in saline, clumped and precipitated in large membranous flakes.

The colonies formed on the surface of agar plates were rough, granular and irregular, and were larger, under these circumstances, than colonies of the MS, or NS forms.

This form of variant was designated as MR (motile rough).

3. Compact Colonies (Nonmotile Smooth).—The compact colonies had the appearance of small dots (Fig. 3). The average diameter of a colony incubated at 37°C. for 24 hours was from 0.2 to 0.4 mm. Under low power magnification the colonies were yellowish in color. The surface of the young colonies was smooth, while that of colonies more than 48 hours old was slightly uneven.

TABLE I

Comparison of the Four Forms of Hog Cholera Bacillus

	MS	MR	NS	NR
Deep colony in semi-solid medium	homogeneous 2–3 cm.	irregular 1–3 mm.	compact 0.2–0.4 mm.	compact 0.2–0.4 mm.
Surface colonies on agar plates	smooth	rough	smooth	rough
Cell distribution in broth culture	homogeneous cloud (diffuse)	flaky sediment (clumped)	homogeneous cloud (diffuse)	flaky sediment (clumped)
Motility	motile	motile	nonmotile	nonmotile
Precipitation in NaCl and $MgCl_2$ solution	diffuse	clumped	diffuse	clumped
Resistance to normal rabbit serum	not killed	killed	not killed	killed

The bacilli of this form did not clump and were nonmotile. Like the MS bacilli, these produced a homogeneous, cloudy growth in broth and the growth from an agar slant could be easily suspended in normal saline solution. On the surface of an agar plate the colonies were round and smooth, and were indistinguishable from those of the MS type.

This form of variant was designated as NS (nonmotile smooth).

4. Compact Colonies (Nonmotile Rough).—When bacilli from the NS form of colony were cultivated in broth at 37°C. for several weeks and again plated in semi-solid medium, the same type of compact colony appeared. After this procedure, however, when the bacilli

were transferred to broth the growth in some of the tubes was different. Instead of the usual homogeneous, cloudy, growth clumps formed which precipitated. The bacteria from these clumps were nonmotile. In the semi-solid medium compact colonies were formed which were indistinguishable from those of the NS form. When an agar slant culture of these organisms was suspended in normal saline solution, the growth precipitated in large flakes. Surface colonies on agar plates were rough, granular and irregular, and were indistinguishable from the MR form.

TABLE II

Reaction of the Four Forms of Hog Cholera Bacillus in Magnesium Chloride Solution

Culture	Time, hrs.	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	H_2O
MS	2	−	−	−	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−	−	−	−
MR	2	−	+	+	+++	+++	+++	+++	−	−	−
	24	+	+++	+++	+++	+++	+++	+++	±	−	−
NS	2	−	−	−	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−	−	−	−
NR	2	++	++	+++	+++	+++	+++	+++	+++	−	−
	24	+++	+++	+++	+++	+++	+++	+++	+++	−	−

This form was designated as NR (nonmotile rough).

The characteristics of the 4 forms described above are tabulated in Table I.

In plating the old broth culture as described above, the homogeneous (normal MS) colonies predominated. The irregular and compact colonies comprised roughly speaking, 1 or 2 per cent of the total.

Further Differential Tests

Salt Sensitiveness.—Arkwright (10) demonstrated that R forms of *B. typhosus* and *B. dysenteriae* agglutinated spontaneously in 0.85 per cent saline solution while the S types did not. This test was used to distinguish the R from the S bacteria. In the present work, the test was performed as follows:

Solutions of magnesium chloride and of sodium chloride were used as shown in Tables II and III. Bacteria from 24 hour agar slant cultures of each of the 4 forms were suspended in distilled water. This suspension was added to the various dilutions of the series of the 2 salt solutions and incubated at 50°C. for 2 hours. The reactions were observed at the end of 2 hours and again after the tubes had remained in the refrigerator for 24 hours.

TABLE III

Reaction of the Four Forms of Hog Cholera Bacillus in Sodium Chloride Solution

Culture	Time, hrs.	M/1	M/2	M/4	M/8	M/16	H_2O
MS	2	−	−	−	−	−	−
	24	−	−	−	−	−	−
MR	2	+	+	−	−	−	−
	24	+	++	++	++	−	−
NS	2	−	−	−	−	−	−
	24	−	−	−	−	−	−
NR	2	+	+	+	+	−	−
	24	+++	+++	+++	+++	−	−

− = no precipitation.
\+ = moderate precipitation.
++ = marked precipitation.
+++ = complete precipitation, supernatant fluid clear.

The difference in behavior of the 4 forms under these conditions are shown in Tables II and III. The MR and NR forms were clumped by both magnesium chloride and sodium chloride while the MS and NS forms were not. The magnesium chloride was more effective than the sodium chloride.

Survival in Normal Rabbit Serum.—Since the R forms of certain other bacteria are more subject to phagocytosis and destruction than the S forms, attempts were made to test the difference in resistance of the 4 forms of hog cholera bacillus to the bactericidal action of normal rabbit serum.

Broth cultures of the 4 forms of hog cholera bacilli were diluted

1:1,000,000 in saline. One part of each suspension was mixed with five parts of normal rabbit serum in small tubes. A control set of the bacterial suspension was mixed in normal saline solution. The tubes were incubated at 37°C. for an hour after which 0.2 cc. from each tube was plated. The number of colonies on each plate was counted after 24 hours incubation.

Organisms of the MS and NS forms were resistant to the serum and many colonies appeared on the plate, but only a few of the bacteria of the MR and NR forms survived the effects of the serum, and far fewer colonies appeared when these forms were plated. The results are shown in Table IV.

TABLE IV

Comparative Resistance of the Four Forms of Hog Cholera Bacillus to the Bactericidal Action of Normal Rabbit Serum

Culture suspended 1 hour at 37°C. in	Number of colonies on agar plates after 24 hours incubation			
	MS	MR	NS	NR
Rabbit serum	299	2	353	87
Saline	223	202	292	215

Resistance to Carbolic Acid.—The difference in resistance of the 4 forms of hog cholera bacilli to the bactericidal action of phenol was not decisive. Bacilli of the smooth, MS or NS forms were, however, somewhat more resistant than those of the rough, MR or NR forms.

Other tests when applied to the 4 forms failed to show any striking differences. Bacilli from all 4 forms were Gram-negative, short rods. All forms fermented dextrose, mannite, maltose and xylose and produced acid and gas. Lactose, sucrose and arabinose were not fermented. The action on dulcite was irregular, differences in the rate of fermentation appearing even in a series of tubes inoculated with bacilli derived from a single colony. Indol and hydrogen sulphide were not produced by any of the bacilli.

DISCUSSION

For the differentiation of the original form of hog cholera bacillus and its 3 variant forms both semi-solid and solid agar media were

employed. It is obvious that the examination of the colonies produced by each of the 4 forms of bacilli on the surface of agar plates alone, serves only to distinguish broadly the smooth from the rough strains. The dry surface of the agar inhibits migration of the motile bacilli and prevents contrast between the motile and nonmotile forms. For this reason, among the surface colonies on agar, it was only possible to differentiate the smooth (MS, NS) forms from the rough (MR, NR) forms. Even under these conditions the differences between the S and R forms were often not decisive. Further microscopic tests are necessary to determine motility. No differences were detected macroscopically between the MR and the NR colonies on the one hand and between the MS and NS colonies on the other.

When colonies of the 4 forms grow under the surface in semi-solid media motile and nonmotile characteristics appear. The semi-solid medium permits migration of the motile bacilli which form large diffuse colonies while the nonmotile bacilli form minute compact colonies. Thus it follows that the motile smooth (MS) colonies, which on agar surfaces appeared like the nonmotile smooth (NS) colonies, now become many times larger than the NS colonies due to the centrifugal migration of the motile (MS) bacilli. At the same time the semi-solid medium permits spontaneous agglutination of the motile rough (MR) bacilli, which form clusters of secondary colonies thus adding another macroscopic differential feature. Differentiation between the 2 forms of small, compact colonies, nonmotile smooth (NS) and nonmotile rough (NR), is not as clear in the semi-solid media as on agar surfaces since neither form is motile. On agar plates the smooth and the rough surfaces of the colonies serve to differentiate the NS from the NR form. Therefore, for a complete differentiation of the 4 forms several tests are desirable: growth on agar plates, growth in semi-solid media, and microscopic tests or growth in broth to determine clumping of the rough forms.

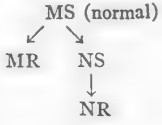
SUMMARY

When the "normal" MS form of hog cholera bacillus was grown for 3 weeks in broth and subsequently plated in semi-solid media, 2 variant forms appeared, a motile rough (MR) form and a nonmotile

smooth (NS) form. The NS form subsequently gave rise to a third variant, namely, the nonmotile rough (NR) form.

It is evident from the observations reported that in the case of the hog cholera bacillus the simple S and R conception of microbic dissociation is inadequate to account for the several variant forms. It appears necessary to combine the S and R with the H and O or the flagellar and somatic antigen conception to account for the phenomenon.

The dissociation is represented by the following diagram:



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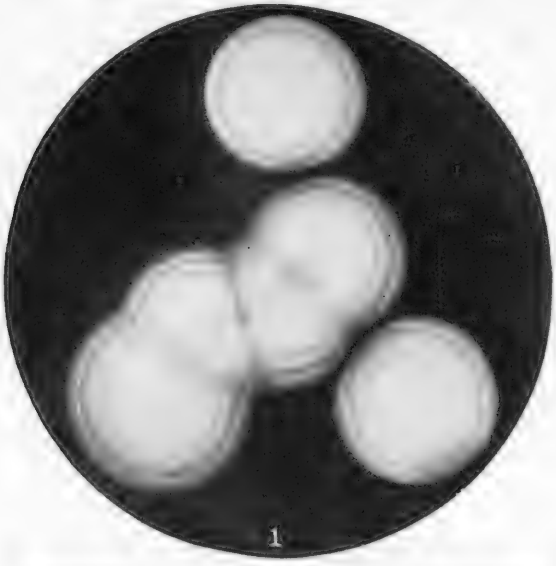
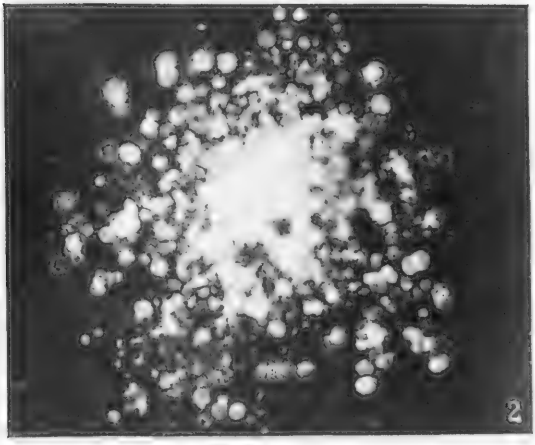
EXPLANATION OF PLATE 12

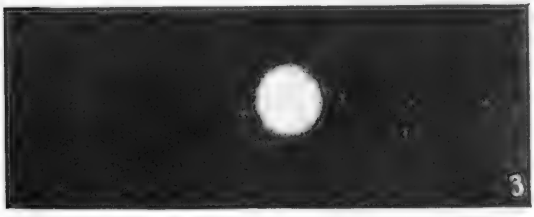
FIG. 1. Normal MS colonies of hog cholera bacilli 17 hours old, growing in semi-solid medium. Natural size.

FIG. 2. MR colony 17 hours old in semi-solid medium, showing the secondary colony formation. × 30.

FIG. 3. NS colony 17 hours old in semi-solid medium. NS and NR colonies appear alike under these conditions. × 30.







(Li: Dissociation of hog cholera bacillus. I)

UNIV. OF

STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

II. Serological Reactions, Virulence and Stability of the Variant Forms

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In a previous paper (1) a study of the dissociation of the hog cholera bacillus was presented. By using both semi-solid and solid agar media the appearance of 3 distinct variant forms was observed arising from the "normal" motile smooth (MS) strain. The 3 variant forms which appeared were designated motile rough (MR), nonmotile smooth (NS), and nonmotile rough (NR). Briefly the appearance of the colonies of the 4 forms which grew deeply in the semi-solid medium was as follows: (a) MS,—large homogenous colonies, (b) MR,—smaller irregular colonies, (c) NS,—minute compact colonies and (d) NR,—minute compact colonies. To differentiate the latter 2 forms macroscopically, it was necessary to observe the colonies as they grew on the surface of agar plates. Under this condition, the smooth surfaces of the NS colonies were distinguished from the rough surfaces of the NR colonies.

The bacteria from the S colonies, both motile (MS) and nonmotile (NS), grew diffusely when cultivated in broth and remained in diffuse suspension when placed in saline solutions. Bacteria from the R colonies, both motile (MR) and nonmotile (NR), grew in clumps in broth media and when suspended in salt solutions clumped and precipitated in flakes. It was also observed that bacilli from both S forms of colonies were more resistant to the bactericidal action of phenol and of normal rabbit serum than the organisms derived from the R colonies.

In the present report further differential tests were applied to

the 4 forms of the hog cholera bacillus. A study of the serological reactions, of the virulence and of the stability of the 4 forms was made.

Serological Reactions

Reference was previously made (1) to a schematic diagram by White (2) representing the dissociant forms of the paratyphoid group of bacteria. The probability that the flagellar and somatic antigens were identical with the H and O antigens respectively was also remarked. Under this assumption the 4 forms of hog cholera bacillus, MS, MR, NS and NR may be represented by the symbols OH, ØH, O and Ø respectively (Ø = the rough form of O). Accordingly, the theoretical serological reactions of the various forms are shown in Table I. A study of the actual serological reactions was then made.

TABLE I

Theoretical Agglutination of the Variant Forms of Hog Cholera Bacilli Assuming that MS = OH, MR = ØH, NS = O and NR = Ø

Antigen	Immune sera			
	Anti-MS (OH)	Anti-MR (ØH)	Anti-NS (O)	Anti-NR (Ø)
MS (OH)	+	+	+	−
MR (ØH)	+	+	−	+
NS (O)	+	−	+	−
NR (Ø)	−	+	−	+

+ = agglutination.
− = no agglutination.
O = smooth, heat stable, granular, somatic antigen complex.
H = heat labile, floccular, flagellar antigen complex.
Ø = rough, heat stable, granular, somatic antigen complex.

Methods

Preparation of Sera.—For the preparation of the immune sera, bacteria from agar slant cultures were suspended in salt solution and killed at 56°C. Rabbits were then immunized by injecting suspensions of the 4 forms of bacilli intravenously 6 or 7 times at 5 to 7 days intervals. A week after the last injection the animals were bled to death and the serum was obtained.

Sera were absorbed with heavy suspensions of living bacteria in 0.1 per cent salt solution (stronger solution of salt caused spontaneous clumping of the R forms and was not used). The mixtures were incubated at 37°C. for 2 hours, placed in the ice box for 24 hours and finally centrifuged to regain the diluted serum. The final dilution of the sera was 1:20.

Agglutination Tests.—The bacteria to be tested were derived from agar slant cultures and were suspended in 0.1 per cent salt solution. Dilutions of sera in 0.1 per cent salt solution, varying from 1:40 to 1:10240, were made to which the bacterial suspensions were added. Suspensions of bacteria in 0.1 per cent salt solution served as control tests. The tubes were then incubated at 50°C. for 2 hours. Readings were made at the end of this time and again after the tubes had been in the ice box for 24 hours.

TABLE II

Agglutination Reactions Actually Observed

Culture tested	Agglutination titer			
	Anti-MS serum	Anti-MR serum	Anti-NS serum	Anti-NR serum
MS	5120 F	5120 F	640 G	—
MR	5120 F	5120 F	160 ?	160 G
NS	640 G	—	640 G	—
NR	—	160 G	—	160 G

F = floccular.
G = granular.
— = no agglutination.
? = differing from theoretical result.

The results of the agglutination tests are shown in Table II. The figures represent the highest dilution in which macroscopic agglutination occurred. The letter F indicates a floccular type of precipitation and G indicates granular precipitation.

It is evident that the actual agglutinations represented in Table II correspond closely to the theoretical reactions predicted in Table I. An exception is noted by the agglutination of MR bacteria in a relatively low dilution of anti-NS serum. The reason for this is not clear and is at present unexplained. It is possible that the anti-NS serum contained some H agglutinins which was indicated by the fact that after the MR bacilli were heated to 100°C. for

30 minutes to destroy the H antigen, no agglutination in anti-NS serum occurred.

The titer of the anti-NR serum was low and attempts to produce a stronger anti-serum were not successful.

Different types of agglutination were observed as indicated in the table. Bacteria of the MS and MR forms when agglutinated by either anti-MS or anti-MR sera promptly formed large, loose, floccular clumps, due to the presence of the H antigen and antibody. MS bacilli in anti-NS serum and MR bacilli in anti-NR serum

TABLE III

Results of Absorption Tests

Bacterial suspensions	Anti-MS (OH) serum			Anti-MR (ØH) serum			Anti-NS (O) serum			Anti-NR (Ø) serum	
	Unabsorbed	Absorbed with MR bacilli	Absorbed with NS bacilli	Unabsorbed	Absorbed with MS bacilli	Absorbed with NR bacilli	Unabsorbed	Absorbed with MS bacilli	Absorbed with MR bacilli	Unabsorbed	Absorbed with MR bacilli
MS (OH)	5120 F	640 G	2560 F	5120 F	—	5120 F	640 G	—	640 G		
MR (ØH)	5120 F	—	2560 F	5120 F	160 G	5120 F	160 ?	—	—	160 G	—
NS (O)	640 G	640 G	—				640 G	—	640 G		
NR (Ø)				160 G	160 G	—				160 G	—

The figures represent the highest dilution in which macroscopic agglutination occurred.

F = floccular agglutination.

G = granular agglutination.

? = differing from theoretical result.

slowly formed into hard, small, granule-like clumps. Bacteria of the NS and NR forms formed similar small granular clumps whenever agglutinated by any of the 4 anti-sera, due to the presence of the O or Ø antigen and O or Ø antibody.

Absorption Tests.—The results of absorption tests which are given in Table III confirmed the results obtained by the ordinary agglutination reaction. The floccular H agglutinin was absorbed from the anti-MS (OH) serum by the MR (ØH) bacilli, but the granular (O) agglutinin remained intact. MR (ØH) bacilli absorbed the

granular (Ø) antigen from the anti-NR (Ø) serum. Anti-NS (O) serum was unaffected by MR (ØH) bacilli.

Similarly the MS (OH) bacteria exhausted the floccular (H) agglutinin from the anti-MR (ØH) serum as well as the granular (O) agglutinin from the anti-NS (O) serum, since these bacilli possess both O and H antigens. The granular (Ø) agglutinin in the anti-MR (ØH) serum was, of course, unaffected.

Lastly NS (O) bacteria had no influence on the H agglutinin in anti-MS (OH) serum and the NR (Ø) organisms absorbed the

TABLE IV

Virulence of the Four Forms of Hog Cholera Bacilli for Hamsters (Chinese Field Mice)

Culture tested	Dose, cc.	No. used	No. died	Mortality
MS	0.1	5	5	100%
	0.001	10	10	100%
	0.000001	5	4	80%
MR	0.1	5	0	0
	0.1	10	0	0
NS	0.1	5	5	100%
	0.001	10	5	50%
NR	0.1	5	0 (abscess)	0
	0.1	10	0 (abscess)	0

Animals were observed for 30 days.

granular (Ø) agglutinins from the anti-MR (ØH) serum but left its H agglutinin intact.

It should be mentioned that for the various serological tests only typically reacting bacteria were used. Indefinite, intermediate, or confusing forms which reacted atypically were frequently encountered and were discarded.

Virulence

Besides the differences in the serological reactions of the 4 forms of the hog cholera bacillus, striking differences in virulence for hamsters (Chinese field mice) and rabbits were observed. Varying

amounts of 24 hour old broth cultures of the 4 forms of bacilli were injected subcutaneously into hamsters. The results of the experiment are given in Table IV.

From the table it appears that in general, as in other bacterial species, the S forms were virulent and the R forms were not. The virulence of the MS form seems to be somewhat greater than that of the NS form. The NR form, however, can probably not be considered to be entirely avirulent. Hamsters receiving 0.1 cc. doses of the NR form subcutaneously occasionally died and abscesses usually developed at the site of inoculation in those that survived. The MR form on the other hand produced no gross pathological changes. Both the MR and the NR forms were frequently virulent for hamsters when injected in large doses (0.5 cc.) intraperitoneally.

Rabbits, when inoculated with the 4 forms of bacilli, reacted in a similar manner. The MS form caused death in 7 days after the subcutaneous inoculation of 0.000001 cc. of broth culture. Of 2 rabbits inoculated with 0.1 cc. of an NS culture, one died after 9 days and the other recovered after a period of fever lasting about 3 weeks. Both NR and MR organisms failed to kill rabbits even in 0.1 cc. doses, but the animals receiving NR bacilli, as in the preceding experiment, developed fever (40°C.) and appeared ill for several days but recovered.

Cultures were made from the spleen of all animals which succumbed during the course of the virulence tests. The same forms of variant bacilli as those used for inoculation were recovered.

Studies on the Stability or Reversibility of the Variant Forms

Reversion in Broth.—Attempts were made to induce reversion from one form to another. The 3 variant forms were repeatedly grown in meat infusion broth. Transfers were made twice daily for 32 days. At intervals during this procedure the cultures were plated in semi-solid media and on the surface of agar plates, and examined. The bacteria were also tested for motility and for clumping in salt solution.

During the experiment, the NR and the NS forms invariably reproduced only their respective kinds. No reversion occurred. However, after several transfers of the MR form, many MS colonies

of the original "normal" strain appeared among the MR colonies. Certain MR forms therefore reverted to the original MS form.

Effect of Animal Passage.—Broth cultures of the 3 variant forms were injected intraperitoneally into hamsters. Large doses of MR and NR strains were virulent when injected intraperitoneally instead of subcutaneously. The 3 strains were passed through 15 mice by intraperitoneal injection of peritoneal washings from mice killed by the bacilli. At intervals during this procedure the bacilli were plated in semi-solid medium and on solid agar, and the colonies were examined.

The NS and NR forms were unaffected by mouse passage and did not revert to any other form. The MR form, on the other hand, showed a tendency to revert to the MS form. After the 6th passage a few MS colonies appeared among many MR colonies. During subsequent (15) passages the proportion of MS to MR colonies gradually increased.

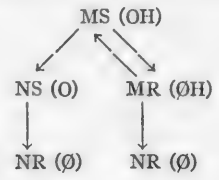
Effect of Growth in Immune Serum.—Since other bacteria have been found to revert from R to the S form during growth in anti-R sera, similar trials were made with hog cholera bacilli. The 3 variant forms NS, MR and NR were transferred every other day in broth containing 10 per cent of NS, MR and NR anti-sera respectively for 2 weeks. The cultures were plated and examined at intervals. The NS form showed further dissociation into the NR form. NR bacilli also appeared among the MR forms during the transfers. The NR forms arising from the MR form were identical with the NR forms which were derived from the NS bacilli. The NR form remained unchanged. It appeared from this test that growth in anti-R sera did not cause a reversion of the NR or MR forms to the original form but did cause further dissociation of the MR and NS organisms.

SUMMARY

The experiments recorded in the present paper confirm the existence of 4 forms of the hog cholera bacillus described in a previous paper (1), namely the "normal" type strain MS and its 3 variants, MR, NS and NR. Serological evidence is also presented to show that the symbols MS, MR, NS and NR represent the similar

conceptions of previous investigators who have used the letters OH, O, ØH and Ø respectively to designate variant forms of other organisms. It has been shown that the S form of the hog cholera bacillus, as the S forms of other bacteria, is more virulent for laboratory animals than the R forms.

In regard to the reversibility of one form to another it was found that by transfer in broth or by passage through mice the MR form showed a tendency to revert to the original MS form. Transfer in broth and animal passage, however, failed to induce any variations in the NR or NS forms. Growth in homologous immune sera did not cause reversion to original forms but in fact provoked further dissociation of the MR to the NR form and also of the NS to the NR form. The MR form may revert to the original normal MS form or may dissociate further into the NR form and is, therefore, the most unstable of the variants. Further attempts to induce changes in the other variants were not made. The dissociation as observed may be represented thus:



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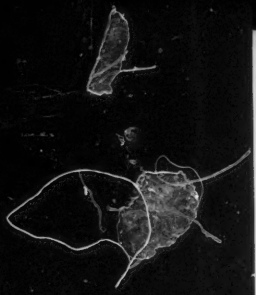

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